THE BIOCHEMICAL JOURNAL

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Medical

Nomenclature of Amino-acids

The following rules have been agreed upon between the Editors of the *Journal of the Chemical Society* and of the *Biochemical Journal*, after consultation with their American colleagues. The examples are given solely to illustrate the application of the rules; in other respects (e.g. the method of enumeration of carbon atoms) names will naturally follow the customary usages of the journal in which they are to appear.

RULE 1. In cases where no possibility of misunderstanding can arise regarding which isomer of the amino-acid is under discussion, the amino-acid may be named without prefix or symbol to denote the direction of the rotation.

RULE 2. In cases where distinction between the stereoisomers is necessary, or considered desirable for the sake of clarity, the configurational family to which the *alpha carbon atom* belongs should be designated by a prefixed *small capital letter*. An additional sign to denote the direction of the rotation is not necessary.

Examples: L-leucine, D-valine.

The optically inactive mixture or racemic compound should, except where Rule 1 applies, be designated with the prefix DL.

Example: DL-leucine.

RULE 3. The configuration of carbohydrates and other compounds which can be configurationally related to glyceraldehyde is designated by a prefixed D or L. Where confusion is possible between the use of the small capital letter prefix for the configuration of the *alpha carbon atom* in amino-acid nomenclature and for that of the highest numbered asymmetric carbon atom in carbohydrate nomenclature, a subscript is added to the small capital letter prefix. Where the prefix is used in the amino-acid sense, the subscript 's' is added; where the prefix is used in the carbohydrate sense, the subscript 'g' is added. These subscripts refer, respectively, to serine, the fundamental substance to which amino-acids that bear structural resemblance to the carbohydrates can be formally related, and to glyceraldehyde, the fundamental substance to which the configuration of the carbohydrates is formally related.

Examples: L_s-threonine, for which the synonym in carbohydrate nomenclature is 2-amino-2:4-dideoxy-D_g-threonic acid; D_s-threonine, for which the synonym is 2-amino-2:4-dideoxy-L_g-threonic acid; L_s-*allo*threonine, for which the synonym is 2-amino-2:4-dideoxy-L_g-erythronic acid; D_s-*allo*threonine, for which the synonym is 2-amino-2:4-dideoxy-D_g-erythronic acid.

Papers that deal with both carbohydrates and amino-acids and which make use of the prefixes should employ subscripts at least for the names of the amino-acids. The acids derived from amino sugars should be named in conformity with carbohydrate nomenclature but with the use of the subscript: e.g. D_g-glucosaminic acid for 2-amino-2-deoxy-D_g-gluconic acid.

RULE 4. If the configurational relationship of the *alpha carbon atom* has not been definitely established, the actual direction of the rotation in a specified solvent, preferably of the free amino-acid in water, is designated by the prefixes *dextro* or *laevo* or, alternatively, by a plus or a minus sign enclosed in parenthesis.

Examples: *dextro*hydroxytryptophan, (+)-hydroxytryptophan.

RULE 5. The prefix *meso* should be used for the *meso* isomers of amino-acids and their derivatives that are optically inactive because of internal compensation.

Example: *meso*lanthionine, *meso*cystine.

RULE 6. Where the amino-acid has two asymmetric centres, but where internal compensation is impossible, the form which occurs in proteins should be called the L-amino-acid, and the form which has opposite configuration on both asymmetric carbon atoms should be designated the D-amino-acid. The pair having opposite configurations on one asymmetric centre only should be designated D-*allo* and L-*allo*, D and L referring as usual to the *alpha carbon atom*.

Examples: D-*allo*threonine, L-*allo*hydroxyproline.

RULE 7. Derivatives and salts of amino-acids and peptides should, where necessary, be named with the use of D or L to denote the configurational family of the *alpha carbon atom* or *atoms*, the customary rules being otherwise observed.

Examples: acetyl-L-tryptophan, L-histidine monohydrochloride monohydrate, copper L-aspartate, D-leucyl-D-valine.

RULE 8. Where a trivial name is applied to a compound that contains two alpha amino carboxylic acid groupings attached to dissimilar carbon chains, the configurational designation is that of the *alpha carbon atom* of the *smaller of the two chains*.

Examples: D-cystathionine for *S*-(D-β-amino-β-carboxyethyl)-D-homocysteine, L-*allo*cystathionine for *S*-(L-β-amino-β-carboxyethyl)-D-homocysteine.

It is emphasized that the introduction of new trivial names should be avoided.

The Liberation of Glucuronic Acid from Conjugated Glucuronides by β-Glucuronidase

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Department of Biochemistry, University of Edinburgh

(*Received* 18 *April* 1947)

Aqueous extracts of many animal tissues have been found to contain an enzyme, 'β-glucuronidase', which catalyzes the hydrolysis of β-glucuronides. It has been suggested (Oshima, 1934; Fishman, 1940; Fishman & Fishman, 1944) that the production of conjugated glucuronides *in vivo* is due to this enzyme. The activity of β-glucuronidase preparations has usually been determined by measuring the reducing material formed on incubation with a glucuronide (Masamune, 1934; Oshima, 1934, 1936; Fishman, 1939, 1940; Fishman & Fishman, 1944; Graham, 1946; Mills, 1946). The use of such methods rests on the assumption that the hydrolytic action of the enzyme is confined to rupture of the glucosidic link. Evidence for this is indirect, and is confined to experiments by Masamune (1934), who determined colorimetrically the free menthol in enzyme hydrolysates of its glucuronide and found it to correspond to the reducing material produced when the latter was calculated as glucuronic acid. It was considered that this point required further investigation in view of the discovery by Lipschitz & Bueding (1939) that synthesis of glucuronides by liver slices is stimulated, not by glucuronic acid, but by certain C_3 compounds, such as pyruvic acid.

In the first series of experiments described below, glucuronic acid liberated from menthylglucuronide by β-glucuronidase was separated from unchanged substrate and determined by means of the colour reaction with naphthoresorcinol. The results agreed with estimates of free glucuronic acid obtained by reduction of ferricyanide, suggesting that the hydrolysis proceeds according to the generally accepted view. Since, however, the colour reaction with naphthoresorcinol is not specific for glucuronic acid (Mandel & Neuberg, 1908; Dische, 1947), a second series of experiments was carried out in which the method of Lohmar, Dimler, Moore & Link (1942) was used to isolate and identify the uronic acid.

EXPERIMENTAL

General methods

Biosynthetic *l*-menthyl-D-glucuronide (Williams, 1938) was used as substrate in all the experiments. β-Glucuronidase was prepared from beef spleen according to the directions given by Graham (1946). Unless stated to the contrary, the enzyme was highly purified (stage *G* in Graham's method), and had a negligible 'reducing blank', even after incubation. Reducing material in hydrolysates of menthylglucuronide was estimated by the procedure previously described (Levvy, 1946). The determination of free glucuronic acid and of menthylglucuronide by means of the colour reaction with naphthoresorcinol was done by the method of Hanson, Mills & Williams (1944). In agreement with these authors, D-glucurone and menthylglucuronide were found to be equivalent in colour production.

Free glucuronic acid and reducing material in hydrolysates

Procedure. To 0·5 ml. enzyme in a 10 ml. centrifuge tube were added 1·0 ml. 0·1 M-acetate buffer, pH 5, and 0·5 ml. substrate solution (20 mg. menthylglucuronide/ml., adjusted to pH 5). The tube was stoppered and placed in a bath at 37°. At the end of a predetermined period, protein was precipitated with $CuWO_4$ and $BaCO_3$ (Levvy, 1946), using 1·5 ml. of each of the two solutions specified. After centrifuging, 3 ml. of the supernatant was removed and diluted to 25 ml. From this solution, 1 ml. samples were taken for the determination of reducing material, and 0·4 ml. samples for the colorimetric estimation of total glucuronic acid (after prior dilution to 2 ml.). Free glucuronic acid was determined in 1 ml. samples after acidification with 1 ml. 0·02 N-H_2SO_4 and extraction with peroxide-free ether for 1 hr. (see below). Before developing the colour, the aqueous layer was freed from ether by placing the extraction tube in hot water and blowing air over the surface of the liquid.

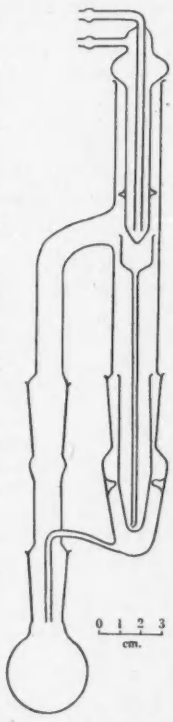


Fig. 1. Micro-liquid-liquid extraction apparatus.

The separation of free and combined glucuronic acid. The removal of unchanged menthylglucuronide from free glucuronic acid by ether extraction was carried out in the specially designed micro-liquid-liquid extractor shown in Fig. 1. The aqueous layer was left at

the end of the extraction in a convenient form for further manipulation, and since the extraction chamber could readily be kept cool a very rapid stream of ether could be maintained through the solution. No difficulties were encountered in the manufacture of the apparatus in spite of the double joint. When the glass tube for return of ether to the flask was softened by heating a good fit at both joints was readily obtained. The course of extraction of known amounts of menthylglucuronide under conditions resembling those in the experiments described above is shown in Fig. 2. Free glucuronic acid was not extracted under these conditions.

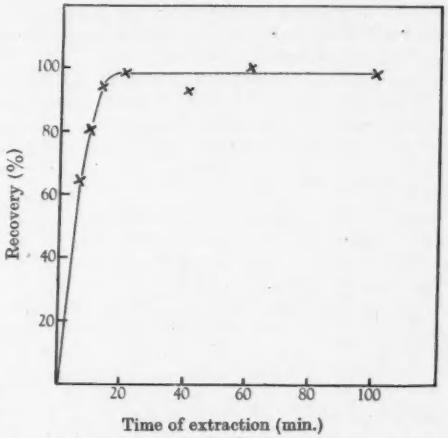


Fig. 2. Extraction of menthylglucuronide with ether.

Results. Fig. 3 shows the course of hydrolysis of menthylglucuronide followed colorimetrically and by ferricyanide reduction. Similar results were obtained with another enzyme preparation. It should be pointed out that the average recovery of glucuronic acid, free and combined, corresponded to only 85% of the total originally added as menthylglucuronide, suggesting retention by the protein precipitate. There would appear, however, to be no doubt that the reducing material in the filtrate was quantitatively accounted for as glucuronic acid.

The isolation of glucuronic acid as the dibenziminazole derivative of D*-glucosaccharic acid*

The hydrolysis. Menthylglucuronide (700 mg.) was dissolved by warming in 10 ml. 0·4N-acetate buffer. When cool, the pH was adjusted to 5 (glass electrode). To the solution was added 10 ml. of an enzyme preparation containing a total of 500 'glucuronidase units' (Graham, 1946). The mixture was kept at 37° for 24 hr., and then dialyzed through Visking sausage casing against 80 ml. water in the refrigerator for a further 24 hr. After acidifying with 2 ml. 11N-H_2SO_4, the dialysate was extracted with ten 60 ml. portions of peroxide-free ether in a separating funnel to remove menthol and unreacted substrate. The aqueous layer was shaken vigorously with 4 g. powdered $BaCO_3$, added in small portions, till the solution was free from sulphate ion, and filtered. The pH of the filtrate, originally about 7·5, was brought to 7 with HCl, and water was removed *in vacuo*. The residue was dissolved in 10 ml. water and, after removal of 0·2 ml. for determinations of

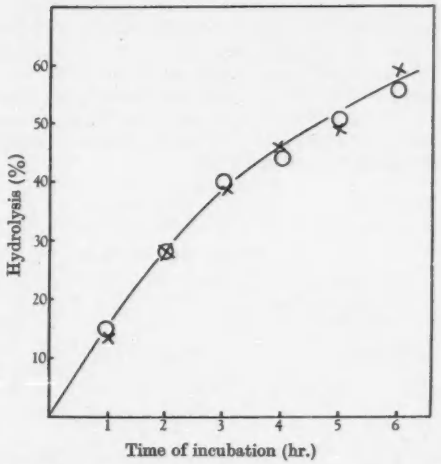


Fig. 3. The enzymatic hydrolysis of menthylglucuronide determined colorimetrically and by reduction of ferricyanide. ×, reduction; o, colour reaction.

reducing material, evaporated once more to dryness. The use of ethanol to remove last traces of water was considered inadvisable in view of the risk of esterification. From its reducing power, the total residue appeared to contain 148 mg. glucuronic acid. When correction is made for glucuronic acid left in the dialysis sac, this corresponds to 49% hydrolysis of the substrate. Control experiments showed that no glucuronic acid was liberated in the absence of enzyme under the conditions of the experiment, and that menthylglucuronide was completely removed from aqueous solution by the extraction process.

Isolation of the dibenziminazole. The residue from the hydrolysis was taken up in 16 ml. water containing 800 mg. barium benzoate. After cooling to 0°, 0·15 ml. bromine was added. The oxidation mixture was kept in the dark at room temperature for 1 day and at 25° for a second day. Excess bromine, ions and benzoic acid were removed (Hudson & Isbell, 1929) and the saccharic acid coupled with *o*-phenylenediamine as described by

Lohmar *et al.* (1942); 60 mg. of the dibenziminazole, m.p. 234–235° (corr.), was obtained (22% of the theoretical in terms of glucuronic acid in the hydrolysis residue).

The hydrolysis was repeated using only 300 units of enzyme, but continuing the incubation for 48 hr. From its reducing power, the residue in this case contained 195 mg. of glucuronic acid (corresponding to 65% hydrolysis) and yielded 105 mg. of the iminazole (30% of theoretical), m.p. 235–236° (corr.). This was combined with the product of the first experiment and put through one stage of purification, giving a final yield of 130 mg. The dihydrochloride and dipicrate were prepared and, as shown by the data in Table 1, the identity of the iminazole with an authentic specimen prepared from D-glucosaccharic acid was proved. Since both the hydrochloride and picrate were found to be hydrated, details of their preparation and figures obtained on analysis are given below.

The hydrochloride. To prepare the hydrochloride, the free base was dissolved in warm N-HCl (0·1 g./ml.). The crystals which separated in the refrigerator were washed with a few drops of ice-cold water and a large volume of acetone. If necessary the hydrochloride was recrystallized from a very small volume of water. After drying *in vacuo* over $CaCl_2$ at room temperature it was obtained as the tetrahydrate. An authentic specimen gave: C, 43·42; H, 5·71% (calc. for $C_{18}H_{20}O_4N_4Cl_2.4H_2O$: C, 43·29; H, 5·62%). After drying for 10 hr. *in vacuo* at 110° over P_2O_5, the loss in weight was 14·63% (calc. for the tetrahydrate, 14·42%). The dried material gave: C, 50·05; H, 5·07% (calc. for $C_{18}H_{20}O_4N_4Cl_2$: C, 50·58; H, 4·68%). Lohmar *et al.* give the m.p. as 257–258° (uncorr.).

The picrate. To prepare the picrate, the hydrochloride was dissolved in hot water and excess picric acid was added as a hot, saturated aqueous solution. The liquid was allowed to cool slowly. In this way, separation as an oil was avoided. The crystals were washed with water and, if necessary, recrystallized from a large volume of water. After drying *in vacuo* over $CaCl_2$ at room temperature, this compound was obtained as the trihydrate. An authentic specimen gave: N, 16·35% (calc. for $C_{30}H_{24}O_{18}N_{10}.3H_2O$: N, 16·17%). After drying for 10 hr. *in vacuo* at 110° over P_2O_5, the loss in weight was 5·79% (calc. for the trihydrate, 6·23%). The dried material gave: C, 44·47; H, 3·06% (calc. for $C_{30}H_{24}O_{18}N_{10}$: C, 44·33; H, 2·96%). Lohmar *et al.* give the m.p. as 211° (uncorr.).

The reducing material in crude enzyme preparations

In the experiments previously described, highly purified β-glucuronidase, containing little if any reducing material, was employed. It was considered of interest to discover whether the reducing material in crude enzyme preparations could be accounted for entirely or in part as glucuronic acid. Two preparations of β-glucuronidase at stage *A* in Graham's (1946) procedure were taken for this purpose. After removal of protein with $CuWO_4$ and $BaCO_3$, both were found to contain very large amounts of reducing substances (equivalent to 0·75 and 0·48 mg. glucuronic acid/ml. respectively) before incubation. After incubation at 37° for 3 hr. in acetate buffer at pH 5 in absence of added substrate, the reducing material in the latter preparation was equivalent to 0·64 mg. glucuronic acid/ml. In no case did the protein-free filtrate give any colour in the reaction with naphthoresorcinol.

Table 1. *Properties of benziminazole derivatives from glucuronic acid*

	Substance isolated	Authentic specimen	Mixture
Dibenziminazole*	m.p. 243°	m.p. 240–242°	m.p. 240°
Dihydrochloride*	m.p. 265°; $[\alpha]_D^{18°} = +52·3$† (S.E. 0·64°)	m.p. 267°; $[\alpha]_D^{16°} = +52·3$‡ (S.E. 0·62°)	m.p. 265–266°
Dipicrate*	Change of form 145°; m.p. 211°; C, 41·83%; H, 3·20% (calc. for $C_{30}H_{24}O_{18}N_{10}.3H_2O$: C, 41·57%; H, 3·47%)	Change of form 145°; m.p. 211°	Change of form 140°; m.p. 211°

* All melting-points corrected. Decomposition before or at melting-point in every case.
† 10·123 mg. tetrahydrate in 0·5 ml. water.
‡ 20·253 mg. tetrahydrate in 1·0 ml. water.

DISCUSSION

From the results of the experiments described above, there would appear to be no doubt that the hydrolytic action of the enzyme known as β-glucuronidase is confined to the rupture of the glucosidic link in conjugated glucuronides. There is thus a distinction to be drawn between 'β-glucuronidase' and the enzyme system which synthesized glucuronides in the experiments of Lipschitz & Bueding (1939), since the latter appeared to build up the hexuronic acid fraction of the molecule from C_2 compounds. In the light of this, it is difficult to accept the postulate of Oshima (1934) and Fishman (1940) that 'β-glucuronidase' is responsible for the production of conjugated glucuronides in the body by a reversal of the hydrolytic action seen *in vitro*. Fishman put forward in support of this view his observation that prior

administration of glucuronidogenic compounds to animals increased the glucuronidase content of certain tissues (Fishman, 1940; Fishman & Fishman, 1944). As an explanation of his experimental data, it would seem at least equally plausible to suppose that 'β-glucuronidase' acts purely as a hydrolytic enzyme in the body, and that increase in the amount of enzyme in an organ occurs in response to the presence of excess glucuronide formed from the aglycone administered. The work of Mills (1946), who found that splenectomized rabbits fed with menthol conjugated larger fractions with glucuronic acid than normal, could be more readily explained by the latter theory.

Since the reducing material formed from menthylglucuronide can be accounted for as glucuronic acid, it appears that estimates of β-glucuronidase activity based on the former are sound. Considerable practical difficulties may, however, be encountered in assaying the enzyme in this way if the preparation is at all impure. The preparation may initially contain large amounts of reducing substances, and these may increase when the enzyme is incubated in absence of substrate (Graham, 1946). Heat inactivation of the enzyme prevents further production of reducing material. Graham therefore recommended that controls in determining glucuronidase activity should consist of enzyme incubated for the same length of time as the reaction mixture, instead of heat-inactivated enzyme as used by Fishman (1939). Graham's procedure would not, however, be correct if the reducing material in the controls were derived from a glucuronide, since in the experimental tubes the latter would merely add to the excess substrate. Enzyme preparations with very high reducing powers were found to give completely negative reactions with naphthoresorcinol, indicating the absence of glucuronic acid, free or in combination as a glucuronide. It appears safe to assume that production of the unknown reducing substances will proceed at the same rate in experimental and control tubes, independently of the enzyme's action, and that Graham's procedure is therefore justifiable.

SUMMARY

1. The reducing material formed when menthylglucuronide was incubated with freshly purified beef spleen β-glucuronidase was accounted for by free glucuronic acid, determined by the colour reaction with naphthoresorcinol.

2. The glucuronic acid in enzyme hydrolysates of menthylglucuronide was isolated and characterized as the dibenziminazole derivative of D-glucosaccharic acid.

3. Crude preparations of β-glucuronidase, containing large amounts of reducing material, did not give the colour reaction for glucuronic acid and conjugated glucuronides.

4. The theoretical implications of these findings are discussed.

REFERENCES

Dische, Z. (1947). *J. biol. Chem.* **167**, 189.
Fishman, W. H. (1939). *J. biol. Chem.* **127**, 367.
Fishman, W. H. (1940). *J. biol. Chem.* **136**, 229.
Fishman, W. H. & Fishman, L. W. (1944). *J. biol. Chem.* **152**, 487.
Graham, A. F. (1946). *Biochem. J.* **40**, 603.
Hanson, S. W. F., Mills, G. T. & Williams, R. T. (1944). *Biochem. J.* **38**, 274.
Hudson, C. S. & Isbell, H. S. (1929). *Bur. Stand. J. Res., Wash.*, **3**, 57.
Levvy, G. A. (1946). *Biochem. J.* **40**, 396.
Lipschitz, W. L. & Bueding, E. (1939). *J. biol. Chem.* **129**, 333.
Lohmar, R., Dimler, R. J., Moore, S. & Link, K. P. (1942). *J. biol. Chem.* **143**, 551.
Mandel, J. A. & Neuberg, C. (1908). *Biochem. Z.* **13**, 148.
Masamune, H. (1934). *J. Biochem., Tokyo*, **19**, 353.
Mills, G. T. (1946). *Biochem. J.* **40**, 283.
Oshima, G. (1934). *J. Biochem., Tokyo*, **20**, 361.
Oshima, G. (1936). *J. Biochem., Tokyo*, **23**, 305.
Williams, R. T. (1938). *Biochem. J.* **32**, 1849.

Preliminary Studies on the Carbohydrate-rich Fractions of Ox Serum

By A. M. STAUB and C. RIMINGTON, *University College Hospital Medical School, London, W.C.* 1

(*Received* 7 *March* 1947)

The glycoproteins of blood have formed the subject of numerous researches. Their existence having been once established (Bywaters, 1909; Biery & Ranc, 1914; Rimington, 1929), investigators have attempted either their isolation or the elucidation of the nature of the carbohydrate-containing complex. Attempts at isolation (Meyer, 1938; Sørensen, 1938; Hewitt, 1936, 1937; Rimington, 1931, 1940) have not led to a product which has been characterized as pure by the modern physico-chemical criteria available (e.g. electrophoresis, ultracentrifugation). McMeekin (1940) has recently announced the isolation of a crystalline carbohydrate-containing albumin fraction which was also electrophoretically

homogeneous, but these two criteria alone are insufficient evidence of purity, and we decided therefore to apply to this substance further tests including the rigorous one of constant solubility under a given set of conditions. Unfortunately the procedure of isolation recorded is extremely laborious, and although we have seen crystals answering to the description given by McMeekin we have not been able to obtain workable quantities of the substance.

The constitution of the polysaccharide moiety of the blood glycoproteins has not as yet been unequivocally settled. Are we dealing with a galacto-mannose-glucosamine, a gluco-galactose-glucosamine or even with several different polyosides? Penet (1940), Dumazert & Memmi (1943) and Dumazert, Lévy & Marszak (1944) favour the latter view. Dumazert & Lévy (1943) isolated and attempted to identify a carbohydrate fraction from the plasma which is not present in the free state but in an easily detachable form. This fraction, which they call 'a loosely combined carbohydrate', would be the same in their view as the 'sucre virtuel' of Lépine (1921). It contains no glucosamine but only glucose and might, they suggest, be composed of three molecules of glucose united to one of phosphoric acid. Such a substance would clearly be of considerable physiological interest.

It is with two main problems in mind, purification of starting material and elucidation of chemical constitution, that we have undertaken the more extended study of a glycoprotein, a seromucoid fraction previously obtained by one of us (Rimington, 1940) from serum. In particular we have attempted (1) to explore the solubility curve of our mucoid in the presence of a precipitant, and (2) to cleave the carbohydrate-protein linkage by methods more gentle than acid or alkaline hydrolysis and to investigate the polyosides so obtained.

MATERIAL AND METHODS

Starting material

This was a seromucoid fraction obtained after removal of heat-coagulable protein from ox blood and precipitation by 50% (v/v) ethanol at pH 4. It was thus equivalent to seromucoid fraction *e* described by Rimington (1940, p. 933).

Analytical methods

Total hexose. Both the orcinol method as described by one of us (Rimington, 1940) and the carbazole method of Gurin & Hood (1939) have been employed, the latter affording somewhat more consistent figures. A mixture of equal parts of mannose and glucose served as reference standard except when examining one fraction by the carbazole method when a glucose standard was used (see discussion). Comparison was made in a Pulfrich step photometer, the orcinol colours always being read through both filters S 47 and S 50, whilst for carbazole colours, filter S 50 was employed. Results obtained by the carbazole method were higher by a factor of about 1·4 than those obtained with orcinol but, allowing for this factor, agreement was very good. (Gurin & Hood (1939) have already observed that carbazole reacts to some extent with amino-acids.) In any one series of experiments the same method was used throughout.

Glucosamine. The acetylacetone and Ehrlich reagent technique has been employed under the conditions already described (Rimington, 1940) using filter S 53.

Nitrogen was determined by the micro-Kjeldahl method using selenium oxide catalyst.

Precipitation curves were based upon the specific property phase rule relationship enunciated by Falconer & Taylor (1946). Since the hexose/nitrogen (H/N) ratio is characteristic for a glycoprotein, we have constructed curves expressing (*a*) soluble nitrogen and (*b*) soluble hexose in relation to concentration of precipitant, and (*c*) soluble hexose as a function of soluble nitrogen.

All equilibrations were carried out at 37° when using Na_2SO_4 and at laboratory temperature when using other salts or ethanol. The different weights (or volumes) of precipitants were introduced into Emich micro-filtration vessels and then a constant volume of the seromucoid solution. The vessels were stoppered, and after the required conditions of time and temperature had been established, filtration was effected through the sintered glass disk by applying a positive pressure of nitrogen.

RESULTS

Precipitation curves and fractionation by Na_2SO_4

Preparation of the seromucoid solution

For all experiments, a solution of the starting material was made as follows. To a weighed quantity of mucoid, distilled water was added to yield a 0·2–0·3% (w/v) solution and the mixture dialyzed against distilled water for 2–3 days. It was then filtered through no. 1 Whatman paper, the latter washed with washings of the dialyzing bag and the volume adjusted to 1 g./l., assuming complete solubility of the protein. This technique afforded more complete solution than simple addition of distilled water (Table 1, compare solution 1 with 2, 3 and 4). The solutions which had a pH between 4 and 5 were used without neutralization and afforded very constant values for the ratio hexose/nitrogen (see Table 1).

Table 1. *Analytical data from different seromucoid solutions prepared as described in text from the same raw material*

Solution	Nitrogen (μg./ml.)	Hexoses by orcinol method using glucose-mannose standard (μg./ml.)	Hexose / Nitrogen
1	155	148	1·04
2	200	196	1·02
3	200	190	1·05
4	200	194	1·03

Choice of precipitant

The characteristics of several precipitants were investigated with the following results.

Ethanol. Although in presence of sodium acetate this caused complete precipitation of seromucoid, the concentration zone of precipitation was too restricted for useful application to the purpose in hand; moreover the volatility of ethanol and the unavoidable dilution of the starting material introduced troublesome sources of error.

Magnesium sulphate and alkaline phosphates were found not to precipitate seromucoid.

Ammonium sulphate. Although capable of precipitating seromucid, the use of this salt was abandoned for analytical reasons, since no agent could be found which would remove the protein completely from solution and thus enable the micro-Kjeldahl method to be employed without preliminary dialysis. Seromucoid is neither heat-coagulable nor is it completely precipitable by phosphotungstic acid, hot tungstic acid or trichloroacetic acid.

Sodium sulphate. Both the anhydrous salt and $Na_2SO_4 . 12H_2O$ precipitated at 37° the major part of the seromucoid and below are given the results obtained with the anhydrous salt, all operations having been conducted in a hot room.

Solubility curves in presence of sodium sulphate

Quantities of anhydrous sodium sulphate varying from 1 to 2·75 g. were weighed and then to each were added exactly 5 ml. of a seromucoid solution prepared as described above. Solution was effected as rapidly as possible by warming the vessel and contents in a water bath at 37°, but with mixtures containing a high concentration of salt the solution

Table 2. *Salting out of seromucoid by sodium sulphate; data for solubility curves*

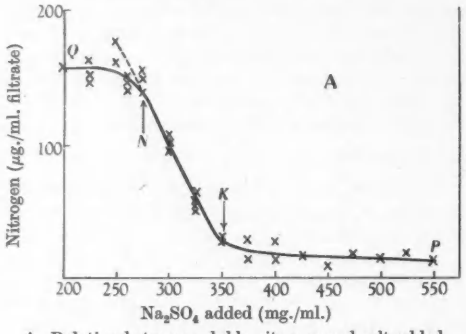
(Two different starting solutions were used (A and B) and the data were obtained in four separate series of experiments performed at different dates indicated by the numbers 1, 2, 3 and 4 respectively.)

Na_2SO_4 added (mg./ml.)	Correction factor $k=(1+x)/b$	Seromucoid solution	Experimental series	Nitrogen in filtrate		Hexoses in filtrate by orcinol method	
				Found (μg.)	Corrected (μg.)	Found (μg.)	Corrected (μg.)
200	1·042	A	1	153	159	160	167
225	1·049	A	1	155	162	168	176
		A	2	144	151	155	163
		A	4	140	147	155	163
250*	1·054	A	1	146	154	155	163
		A	2	152	160	155	163
		B	3	168	177	175	184
260	1·059	A	1	138	146	155	164
		A	2	140	148	150	159
		A	4	132	140	155	164
275	1·062	A	1	132	140	150	159
		A	2	146	155	140	149
		A	4	140	149	150	159
300	1·068	A	1	98	105	120	128
		A	2	96	102	125	133
		A	4	96	102	135	144
		B	3	90	96	120	128
325	1·075	A	1	48	52	85	91
		A	2	50	54	95	102
		A	4	54	58	100	107
		B	3	60	64	102	109
350	1·080	A	1	32	34	82	88
		B	3	30	32	87	94
375	1·087	A	1	16	17	65	70
		B	3	28	30	65	70
400	1·095	A	1	14	15	57	62
		B	3	26	28	55	60
425	1·101	B	3	16	18	50	55
450†	1·104	A	1	9	10	30	33
475	1·104	B	3	16	18	50	55
500	1·104	B	3	14	15	50	55
525	1·104	B	3	17	19	50	55
550	1·104	B	3	12	13	50	55

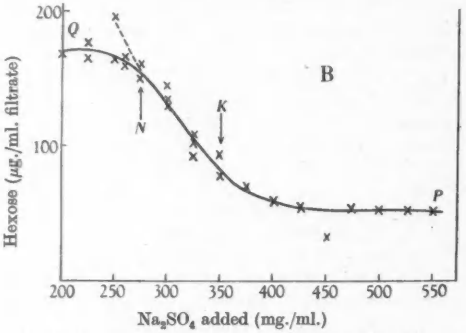
* Ppt. first visible. † Saturation.

was slow and the vessels required periodic shaking for the first few hours. After 24 hr. a precipitate of protein was present in the series which contained a concentration of Na_2SO_4 greater than 250 mg./ml. Filtration was carried out at 37° under a positive pressure of N_2 and samples of 1 ml. of filtrate taken for duplicate nitrogen and hexose determinations. Hexose was frequently determined in triplicate.

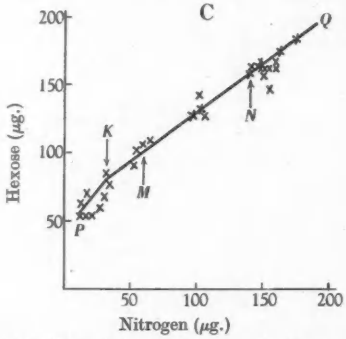
In order that all results should refer to 1 ml. of the original solution of glycoprotein, the experimentally determined values were corrected by a factor k derived from the formula $k=(a+x)/b$, where a = sp.gr. of the original solution of seromucoid, x = weight of Na_2SO_4 added to 1 ml. of this solution and b = sp.gr. of the final solution. In practice, since the solution of seromucoid is very dilute, we have calculated values of k for solutions of Na_2SO_4 of various concentrations in distilled water from the expression $k=(1+x)/b$.



A. Relation between soluble nitrogen and salt added.



B. Relation between soluble hexoses and salt added.



C. Relation between hexoses and nitrogen.

Fig. 1. Salting out of seromucoid by sodium sulphate at 37° (for explanation of lettering see text).

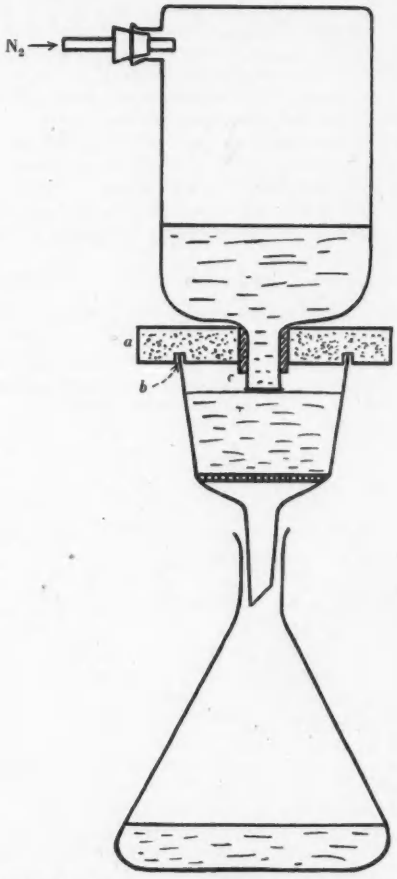


Fig. 2. Filtering device used for fractionation of large quantities of seromucoid by sodium sulphate at 37°. *a*, wooden block; *b*, wax-lined groove; *c*, rubber sleeve.

The results of solubility curve determinations carried out in four series of experiments on different solutions of seromucoid exhibit a satisfactory concordance (Table 2 and Fig. 1). They will be discussed in detail below.

Fractionation of seromucoid by Na_2SO_4

On the basis of information obtained from the solubility curves, a fractionation of seromucoid was carried out as follows: to 450 ml. of the seromucoid solution were added 123·75 g. of anhydrous Na_2SO_4 (0·275 g./ml.) and the mixture kept at 37° for 24 hr., agitation being frequent during the first few hours. Filtration through a large sintered glass filter (porosity G 4) without loss from evaporation was accomplished by the use of a device (Fig. 2) sealing hermetically the solution-container to the body of the filtering funnel. The precipitate was dissolved from the funnel with successive quantities of water and dialyzed, yielding fraction Na I. After further addition of 22·5 g. Na_2SO_4 (0·05 g./ml.) to the filtrate, a further precipitate was similarly removed affording fraction Na II. The final fraction Na III was obtained after a further 78·75 g. Na_2SO_4 (0·175 g./ml.) had been added to the filtrate to achieve full saturation with the salt.

Nitrogen, hexose and hexosamine determinations were made on fractions Na I and Na III but fraction Na II was unfortunately lost before analysis. The analytical results are presented in Table 3 and are further discussed below.

Cleavage of seromucoid by phenol

Seromucoid in the form of a powder was added to 92% (w/v) phenol according to a procedure used by Morgan & Partridge (1941) and dialyzed. The hexose/total N ratio was determined in the water-soluble and water-insoluble fractions afforded by the dialysis.

Fractionation of phenol solutions by ethanol. The solutions of the seromucoid in phenol were either dialyzed directly against distilled water, or were first precipitated by addition of ethanol, the insoluble material being subsequently suspended in water and dialyzed. The latter procedure was more satisfactory, the H/N ratio of the water-soluble fraction after dialysis being higher (Table 4). Moreover this carbohydrate-rich fraction is precipitated completely from the phenolic solution by the addition of 1 vol. of ethanol, further additions of ethanol only bringing down products with a H/N ratio of 0·24–0·70 as compared with 3–4.

Study of the fraction insoluble in water after dialysis of the ethanol-phenol precipitate. A portion of the precipitate was dissolved in a little 0·05N-NaOH, the solution neutralized and dialyzed. Analysis of the solution indicated a fall in the ratio of hexose to nitrogen ($H/N = 0·77$) relative to that of the original (or 'standard') preparation of seromucoid ($H/N = 1·04$). The remainder of the precipitate was washed with water, dried *in vacuo*, powdered and re-extracted by 92% (w/v) phenol. Only a small quantity dissolved and this was worked up as on the former occasion by addition of ethanol, dialysis, etc. The residual water-insoluble material was dissolved in NaOH, neutralized, dialyzed and analyzed as above, but no further decrease of the H/N ratio was apparent. It does not seem possible, therefore, to separate by this means the residual polyoside from

Table 3. *Composition of fractions obtained from seromucoid by fractionation with either sodium sulphate or phenol and chloroform*

Fraction	Nitrogen (%)	Hexoses by carbazole method: (*a*) glucose-mannose standard, (*b*) glucose standard (%)	Glucosamine (%)	Hexose / Nitrogen	Glucosamine / Hexose
Seromucoid	13·0	17·8 (*a*)	8·0	1·44*	0·45
Na I	11·8	11·6 (*a*)	6·7	0·94	0·58
Na III	12·5	15·2 (*a*)	8·7	1·15	0·57
C I	3·9	66·0 (*b*)	10·0	17·0	0·15
C II	11·4	12·4 (*a*)	7·4	1·08	0·60

* The difference from the figure given in Table 1 is due to the use of the carbazole in place of the orcinol method (see text).

Table 4. *Fractions obtained from a solution of seromucoid in phenol by dialyzing directly or by dialyzing after a preliminary ethanol precipitation*

Origin of fraction	Dry wt. (mg./ml.)	Nitrogen (%)	Hexoses by orcinol method: glucose-mannose standard (%)	Hexose / Nitrogen
Dialysis of phenol solution: fraction soluble in water (B)	0·82	8·5	25·0	3
Precipitation of phenol solution by 1 vol. ethanol, ppt. dialyzed: fraction soluble in water (A I)	0·72	7·2	28·5	4

the protein with which it remains in combination in this fraction of the seromucoid.

Purification of the water-soluble fraction after dialysis of the ethanol-phenol precipitate (A I). Two methods were employed, (1) repeated precipitation by ethanol, and (2) agitation with chloroform.

(1) *Ethanol precipitation.* Since a trial fractionation by addition of 1, 2 or 3 vol. of ethanol did not lead to a fraction with appreciably augmented *H/N* ratio, the material was precipitated twice by 5 vol. of ethanol, the final precipitate being completely soluble in water. This fraction A II is characterized by an increase of the *H/N* ratio to 5.

(2) *Agitation with chloroform.* A solution (approx. 0·5 % (w/v)) of the above material A II was shaken repeatedly with chloroform and a few drops of amyl alcohol as described by Sevag (1934). In the first series of experiments the reaction was neutral. When a gel of protein was no longer formed, the material was precipitated by ethanol and redissolved in water yielding fraction A III in which the *H/N* ratio had been raised to 10. The chloroform treatment was continued with fraction A III at an acid reaction (pH 4–5, by addition of HCl) until a thin layer of protein no longer formed at the interface. Fraction A IV was thus obtained which, although containing hexose, contained so little nitrogen that, with quantities available, determinations fell within the range of error of the micro-Kjeldahl method. The ratio *H/N* was subsequently determined upon larger quantities of material (fraction C I, see below), and found to be 17. The above results are collected in Table 5.

Table 5. *Further fractionation of the carbohydrate-rich fraction of seromucoid. Hexoses determined by orcinol method*

Manipulations	Fraction	Hexose / Nitrogen
	Seromucoid	1·04
Solution in phenol, precipitation by 1 vol. ethanol, dialysis	A I	4
Two successive reprecipitations of A I by 5 vol. ethanol	A II	5
Repeated shaking of A II with chloroform and amyl alcohol in neutral solution	A III	10
Repeated shaking of A III with chloroform and amyl alcohol in solution at pH 5	A IV	17

Preparation and analysis of two fractions resulting from seromucoid by phenol cleavage

The application of the results of the small scale experiments to a larger quantity of seromucoid, yielded two fractions C I and C II as follows (see scheme, Fig. 3):

Seromucoid (1 g.) was ground in a mortar with 10 ml. of 92 % (w/v) phenol and the mixture shaken and left for some hours at room temperature, when all dissolved. An equal volume of absolute ethanol was then added and the insoluble fraction separated, suspended in water and dialyzed until free from phenol. The precipitate was collected on the centrifuge, washed with water, dissolved in the minimal quantity of 0·05N-NaOH, then neutralized and again dialyzed, affording fraction C II.

The aqueous mother liquor, to which were added the water washings from the preparation of C II, was treated with 5 vol. of absolute ethanol, the precipitation repeated, the material dissolved in 30 ml. of water, and shaken repeatedly with 6 ml. portions of chloroform until a protein gel no longer formed. The collected chloroform phases were washed with water to remove any non-coagulated protein, and these washings themselves treated with fresh chloroform as above until gel no longer formed, and the aqueous residue united with the main fraction. To the aqueous solution was then added 5 vol. of ethanol, the resulting precipitate was

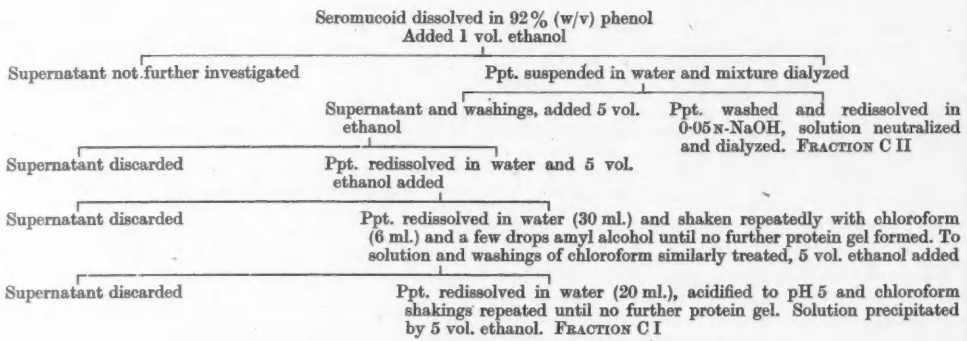

Seromucoid dissolved in 92 % (w/v) phenol
Added 1 vol. ethanol
Supernatant not further investigated
Ppt. suspended in water and mixture dialyzed
Supernatant and washings, added 5 vol. ethanol
Ppt. washed and redissolved in 0·05N-NaOH, solution neutralized and dialyzed. Fraction C II
Supernatant discarded
Ppt. redissolved in water and 5 vol. ethanol added
Supernatant discarded
Ppt. redissolved in water (30 ml.) and shaken repeatedly with chloroform (6 ml.) and a few drops amyl alcohol until no further protein gel formed. To solution and washings of chloroform similarly treated, 5 vol. ethanol added
Supernatant discarded
Ppt. redissolved in water (20 ml.), acidified to pH 5 and chloroform shakings repeated until no further protein gel. Solution precipitated by 5 vol. ethanol. Fraction C I

Fig. 3. Scheme of fractionation of seromucoid by phenol and chloroform.

redissolved in 20 ml. of water, acidified to *c.* pH 5 and again treated with chloroform as already described. A final precipitation with ethanol, resolution and dialysis afforded fraction C I.

Table 3 records the analytical findings upon fractions C I and C II with, for comparison, those derived from the fractions obtained by Na_2SO_4 precipitation.

DISCUSSION

A study of the curves A (nitrogen : concentration of Na_2SO_4), B (hexoses : concentration of Na_2SO_4) and C (nitrogen : hexoses) of Fig. 1 shows that the greater part of the seromucoid is precipitated between the limits of 225 and 425 mg./ml. of Na_2SO_4, the latter figure being close to the figure for full saturation at 37° (440 mg./ml.). It also shows that although the seromucoid studied was not a pure substance (the relationship in curve C would in that case have been represented by a straight line) the amount of impurity was relatively small (compare *KN* with the total curve, Fig. 1 A). To facilitate discussion, we will consider consecutively the four portions of the curves, corresponding to Na_2SO_4 concentrations of 200–275, 275–350 and 350–550 mg./ml., viz. *QN*, *NK* and *KP*, and finally the fraction of the glycoprotein not precipitated by Na_2SO_4.

QN. Our results in this region are indefinite owing to the very fine nature of the precipitates which form in small quantity and are difficult to retain by the G 3 filters employed. On curve C the portion *NQ* may be co-linear with the mean experimental values grouped about *N*, but curves A and B, on the other hand, might suggest that the protein precipitating in this region differs from that precipitating subsequently. Nevertheless the differences between the determinations are often only slightly greater than the limit of experimental error of the methods, and it is not possible to form an unequivocal conclusion concerning this small portion of the seromucoid studied.

NK. All three curves show that over this region it is possible to speak of a characteristic glycoprotein constituting the main fraction of the seromucoid (containing 70% of the nitrogen and 40% of the hexose of the whole material) and having a ratio *H*/*N* of 0·72 (orcinol method) or 1·00 (carbazole method) calculated from curve C.

Electrophoretic analysis at pH 8 of this fraction afforded some confirmation of its homogeneity (Fig. 4), but one must not overlook the fact that the electrophoresis was carried out at one pH only, and furthermore that it is possible by this method to overlook impurities which comprise less than 10% of the main material.

KP. The striking difference in the character of the curves A and B over this region and the much steeper slope of C clearly indicate the presence of a glycoprotein richer in carbohydrate than that previously precipitating and having a ratio *H*/*N* of 1·0 or 1·4 according to whether orcinol or carbazole is used for determination.

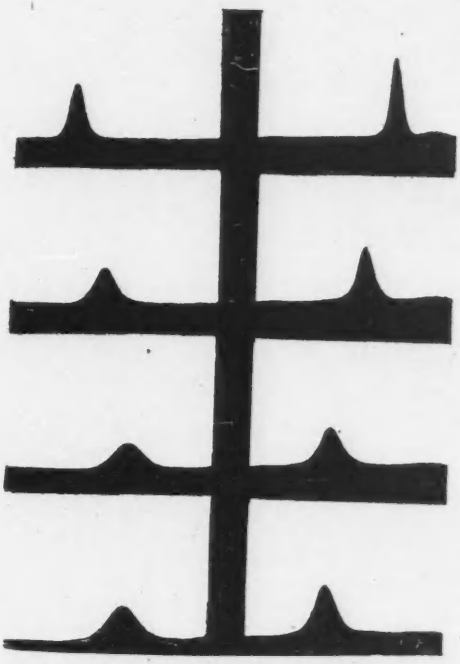


Fig. 4. Electrophoretic pattern of the main fraction obtained from seromucoid by salting out with sodium sulphate. Phosphate buffer, pH 8·0, ionic strength 0·2.

Residual fraction. A fraction representing approx. one-third of the total hexoses and a lesser fraction (approx. one-twelfth) of the total nitrogen remains unprecipitated by Na_2SO_4, the ratio *H*/*N* of this residual material being greater than 3.

An analytical study of fraction Na I (precipitation up to *N*) and Na III (precipitation from *M* to full saturation) agreed with expectations based upon deductions from the three curves (see Table 3). Unfortunately a laboratory mishap deprived us of the supposedly pure fraction Na II (precipitation between *N* and *M*) and we were unable in this instance to confirm by analysis the ratio $H/N = 1{\cdot}0$ deduced from the straight line *MN*. The value found for Na I (0·94) does not differ greatly from this figure, but we are unable to say whether the slight deviation is due to experimental error or to the presence here of a glycoprotein different from Na II and slightly poorer than it is in hexoses. On the other hand the difference between the value of 1·0

for Na II and 1·15 found for Na III is much beyond the possible limits of experimental error and agrees well with the values deducible from curve C.

It is possible indeed to calculate a theoretical value for the ratio H/N for fraction Na III knowing the values of H/N for the portions MK (1·00) and KP (1·4) and the relative proportions of these two materials in the fraction Na III, viz. 2·5/2 deduced from inspection of curve C.

One finds

$$H/N \times 4{\cdot}5 = 2{\cdot}5 \times 1{\cdot}0 + 2 \times 1{\cdot}4, \text{ i.e. } H/N = 1{\cdot}17.$$

The actual value of the ratio H/N, found by experiment was 1·15, an excellent agreement.

In addition to the above results, the study of Table 3 allows interesting conclusions to be drawn concerning the ratio of glucosamine to hexoses in the different fractions. One finds, indeed, an enrichment in glucosamine in Na I and Na III relative to the original seromucoid (0·57 and 0·58 respectively as against 0·45). Moreover these values are the same within the limits of experimental error as that afforded by the fraction C II obtained by phenol cleavage. This enrichment in glucosamine necessitates an impoverishment in the residual fraction not precipitated by Na_2SO_4, if one supposes, as seems reasonable, that fraction Na II would possess the same ratio as Na I and Na III (both of which contain more or less Na II). It would have been interesting to know whether the glucosamine : hexose ratio of the residual fraction, which unfortunately was not kept, was the same as that (0·15) of fraction C I obtained during fractionation with phenol.

It would appear, then, that the seromucoid which we studied contained different proteins united to two different polyosides, one of which was easily detachable by phenol (and perhaps by Na_2SO_4) and poor in glucosamine, whilst the other was more firmly bound to protein and relatively rich in glucosamine. Substantiation is thus afforded of the results obtained by Penet (1940) and Dumazert *et al.* (1943, 1944) and mentioned in the introduction to this paper. It seems indeed possible either that the presence of glucosamine in the easily hydrolyzable polyoside studied by them was not revealed on account of the very small quantities with which they worked, the glycoproteins being examined in the whole plasma, or that the small quantity was destroyed during hydrolysis.

Our determinations of hexose made with carbazole afforded in the case of fraction C I the red colour characteristic of glucose (which finding led us to use a reference standard of glucose while investigating this fraction), whilst all other fractions, viz. Na II, Na III, C II and seromucoid itself invariably gave the brown colour observable when the carbazole method is applied to galactose or to mixtures of glucose and mannose. The few μg. of tryptophan which would be present during these determinations made on glycoproteins could not be responsible for the difference in tint observed. This also accords with the results of Penet (1940) and Dumazert *et al.* (1943, 1944) who found that those carbohydrates which could be liberated by a mild hydrolysis were composed of glucose only.

A chromatographic study of hydrolysates of the two fractions C I and C II would doubtless permit an elucidation of the nature of the hexoses (Partridge, 1946) they contain; we were not able to do more than carry out orientating experiments in this direction owing to the departure of one of us having interrupted the work before definite conclusions could be reached.

CONCLUSION

We have separated from a seromucoid, previously prepared by one of us, a glycoprotein the solubility curve of which entitles it to be regarded as a pure substance, and also a fraction rich in carbohydrate but with a nitrogen content too high to allow it to be classed as a polyoside. This material resembles more closely the group of polyosido-polypeptides familiar to immunologists.

The carbohydrate moieties of these two fractions are distinctly different both as regards their glucosamine contents and the nature of their hexoses. In connexion with the latter one may recall the 'sucre virtuel' of Lépine (1921) which however has never up to the present time been isolated in the form of a polysaccharide from a glycoprotein. Penet (1940) stresses that it is not possible to affirm 'that it involves a linkage to protein'.

SUMMARY

1. Further investigation has been made of a seromucoid fraction previously prepared from ox blood.

2. Salting out studies with sodium sulphate at 37° showed that a fraction could be separated representing about 70 % of the nitrogen and about 40 % of the total bound hexose in the starting material, and satisfying the criterion of constant solubility and composition (hexose/nitrogen) demanded by a pure substance. The homogeneity of this fraction was supported by electrophoresis.

3. Cleavage of a fraction, rich in carbohydrate and very poor in nitrogen, from the seromucoid could be accomplished by fractionation with phenol-ethanol and repeated shaking with chloroform and amyl alcohol at suitable reactions. The residual material was correspondingly poorer in carbohydrate than the parent seromucoid.

4. The ratio of glucosamine to hexoses in these two fractions has been compared with that in the materials obtained by sodium sulphate fractionation. The particularly low ratio of glucosamine to

hexoses in the carbohydrate-rich fraction is emphasized.

5. The possibility is considered that the treatment of our seromucoid with sodium sulphate at 37° also results in the detachment of a carbohydrate-rich fraction.

6. The bearing of these preliminary findings upon current views concerning the nature of the serum glycoproteins is briefly discussed.

We wish to thank Dr R. A. Kekwick of the Lister Institute of Preventive Medicine for kindly performing the electrophoretic examination. One of us (A.M.S.) was the holder of a Fellowship from the French Government and Pasteur Institute during the course of this work.

REFERENCES

Biery, H. & Ranc, A. (1914). *C.R. Acad. Sci., Paris*, **158**, 278.
Bywaters, H. W. (1909). *Biochem. Z.* **15**, 322.
Dumazert, C. & Lévy, M. (1943). *Bull. Soc. Chim. biol.* **25**, 1274.
Dumazert, C., Lévy, M. & Marszak, I. (1944). *Bull. Soc. Chim. biol.* **26**, 1214.
Dumazert, C. & Memmi, A. (1943). *Bull. Soc. chim. biol.* **25**, 1416.
Falconer, J. S. & Taylor, D. B. (1946). *Biochem. J.* **40**, 835.
Gurin, S. & Hood, D. B. (1939). *J. biol. Chem.* **131**, 211.
Hewitt, L. F. (1936). *Biochem. J.* **30**, 2229.
Hewitt, L. F. (1937). *Biochem. J.* **31**, 1534.
Lépine, R. (1921). *Le sucre du sang.* Paris: Librairie Alcan (cited by Penet, 1940).
McMeekin, T. L. (1940). *J. Amer. chem. Soc.* **62**, 3393.
Meyer, K. (1938). *Cold Spr. Harb. Symp. quant. Biol.* **6**, 91.
Morgan, W. T. J. & Partridge, S. M. (1941). *Biochem. J.* **35**, 1140.
Partridge, S. M. (1946). *Nature, Lond.*, **158**, 270.
Penet, G. P. (1940). *Contribution à l'étude des glucides du plasma sanguin.* Marseille: Thèse en pharmacie.
Rimington, C. (1929). *Biochem. J.* **23**, 430.
Rimington, C. (1931). *Biochem. J.* **25**, 1062.
Rimington, C. (1940). *Biochem. J.* **34**, 931.
Sevag, M. G. (1934). *Biochem. Z.* **273**, 419.
Sørensen, M. (1938). *C.R. Lab. Carlsberg*, **22**, 487.

The Reduction of Methaemoglobin in Red Blood Cells and Studies on the Cause of Idiopathic Methaemoglobinaemia

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It is well established that methaemoglobin produced in the blood of normal animals by the injection of drugs disappears quite rapidly on withdrawal of the drug (Dennig, 1900; Stadie, 1921), and that its disappearance is due to reactions taking place in the red cell itself (Cox & Wendel, 1942). The last named authors showed that reduction of methaemoglobin taking place *in vivo* continued at the same speed in the shed blood, and did not depend on reducing substances in the plasma. Drug methaemoglobinaemias in man appear to be closely similar to those in animals, the foreign pigment usually disappearing within 24 hr. after the last dose of the drug.

In idiopathic methaemoglobinaemia, a rare disease which may be familial, it has been found that there is little or no tendency for the methaemoglobin to disappear from the shed blood (Lian, Frumusan & Sassier, 1939; Barcroft, Gibson, Harrison & McMurray, 1945). This observation has recently been confirmed by Sievers & Ryon (1946) who showed that the stability of the methaemoglobin probably does not depend on an abnormality of the plasma, since plasma from their patient did not affect the rate of reduction of methaemoglobin in normal human erythrocytes, nor did normal plasma alter the behaviour of methaemoglobin in erythrocytes from the case.

The work described here has been carried out on blood from six persons belonging to two families in which idiopathic methaemoglobinaemia occurred. It has been possible to obtain evidence of a specific deficiency in the enzyme system of erythrocytes from the cases, and also to draw conclusions concerning the reactions involved in the reduction of methaemoglobin in the blood of normal persons. Apart from their application to the study of idiopathic methaemoglobinaemia, the results are of interest since they throw light on the mechanism by which the haemoglobin of normal persons is maintained in a functional state. The mechanism of regeneration of haemoglobin from methaemoglobin is of importance as it has been suggested that methaemoglobin is constantly being formed in the blood (Peters & Van Slyke, 1931), and Paul & Kemp (1944) have shown that methaemoglobin is present in small amounts in normal human blood.

METHODS

Materials. Hexosediphosphate was prepared by the method of Morgan & Robison (1930). For use, the neutral Ba salt was rubbed up in a small glass mortar with the theoretical amount of N-H_2SO_4, and neutralized with NaOH after removal of $BaSO_4$. Hexosemonophosphate (Robison ester) was isolated from a yeast fermentation mixture as described by DuBois & Potter (1943). Final purification was effected through the Pb salt as described by Robison (1922). As the salt is soluble, Ba was removed at pH 8 as the phosphate. The acid Ba salt of D-3-phosphoglyceric acid was prepared from hexosediphosphate as described by Neuberg & Vercellone (1935). The purity of the specimen of the crystalline salt was checked by the polarimetric method of Meyerhof & Schulz (1938). Na pyruvate was prepared from the freshly distilled acid by the method of Peters (1938) and recrystallized twice from aqueous acetone. Diphosphopyridine nucleotide (DPN) was prepared from brewer's yeast by the method of Green & Brosteaux (1936). Later the isolation of larger quantities from baker's yeast was undertaken, using the simplified method of Green & Williamson (1940). The DPN content of the preparations was determined manometrically by measuring the CO_2 evolution on reduction with hydrosulphite in bicarbonate solution (Warburg, Christian & Griese, 1935). Coenzyme factor was prepared from ox heart in the form of the Straub flavoprotein (Straub, 1939). Purification was carried to the stage of elution from alumina C_γ.

Analytical methods. Venous blood samples of 30–50 ml. were taken from the subjects 2 hr. after breakfast and defibrinated by stirring with glass wool. Before use, the cells were washed three times with 5 vol. 0·9% (w/v) NaCl containing 0·01 M-phosphate buffer pH 7·4, (phosphate saline). It is important that the cells be washed with saline shortly after the blood is drawn, otherwise rapid reduction of methaemoglobin (MHb) may be observed in the controls without substrate.

In the experiments with normal blood and in some of the experiments with blood from the cases of idiopathic methaemoglobinaemia, it was necessary to form MHb in the cells. This was effected by treating the red cells with 3 vol. 0·075% (w/v) amyl nitrite in phosphate saline. The mixture was allowed to stand for 10–15 min. at room temperature, and the cells freed from excess nitrite by repeated washing with phosphate saline. This treatment converts about half of the haemoglobin to MHb. It was noted that in the absence of phosphate the formation of MHb by amyl nitrite takes place much less rapidly. The total pigment in the cell suspensions was usually about 14 g./100 ml. All results, however, have been corrected to refer to a suspension containing 15·6 g./100 ml. to facilitate comparison of the figures in the tables.

The reduction of MHb in the red cells was followed by measuring the uptake of CO consequent on the reduction of MHb to haemoglobin. Samples of 2 ml. of the red cell suspension were measured into the main chamber of Barcroft-Dixon flasks of about 40 ml. capacity. 0·2 ml. 2N-KOH and a roll of filter paper were placed in the inset and substrate added, after equilibration in the water bath, from Keilin tubes. The flasks were filled with 20% (v/v) CO in N_2 by repeated evacuation, the CO being prepared from formic and sulphuric acids. The results in all cases were corrected for the condensation of CO with the KOH in the insets to form formate (Warburg & Christian, 1931). The temperature in all experiments was 37°.

Haemolysates were prepared by washing red cells with 0·9% (w/v) NaCl, and cooling the concentrated corpuscular cream to −2°. Two vol. of ice-cold water were then added, and the mixture allowed to stand for 10 min. A volume of 0·5M-phosphate buffer pH 7·4 equal to that of the blood corpuscles was added and the cloudy liquid centrifuged at high speed for 15 min. The clear supernatant fluid was sucked off and used in the manometers. Pyruvic acid was determined on trichloroacetic acid filtrates of blood by the method of Long (1942). Phosphoglyceric acid was determined by the method of Rapoport (1936). When required, MHb was determined by the method of Evelyn & Malloy (1938), and total pigments by the method of Clegg & King (1942), standardized according to Gibson & Harrison (1945). Where necessary, the quantities of reagents taken for these colorimetric estimations were slightly altered to suit the Hilger Spekker absorptiometer.

RESULTS

Glucose and lactate as substrates in the reduction of methaemoglobin in erythrocytes

The reduction of methaemoglobin (MHb) produced by the action of nitrite on red cells from normal persons is slow when they are suspended in saline. The reduction is greatly accelerated by the addition of glucose (Table 1, cols. A, B). This confirms previous observations of Warburg, Kubowitz & Christian (1930), using rabbit erythrocytes. The effect of lactate is closely similar (Table 1, cols. A, C). Glucose and lactate together do not produce additive effects, which suggests that some stage in the overall reaction is common to both substrates. In the case of lactate it is found that the net molecular ratio *pyruvate formed/MHb reduced* is approximately 0·5 (Table 1, cols. G–E/C–A), in agreement with the equation

$$\text{lactate} + 2\text{MHb} = \text{pyruvate} + 2\text{Hb}. \qquad (1)$$

With glucose as substrate, the position is less clear, since there are several possible pathways for the reaction. The main problem is whether glucose acts before splitting, during glycolysis, into 3-carbon compounds, or after the splitting has taken place. In the first case the reactions would be of the type described by Dickens (1938), involving phosphorylation followed by step-wise oxidation of the molecule through phosphogluconic acid to phosphoketohexonate and decarboxylation to phosphopentose (Fig. 8, steps 1 and 2). The enzymes catalyzing these reactions have been shown to occur in horse blood and require coenzyme II. The other possibility is that reduction of MHb takes place after splitting of the glucose molecule to two triosephosphate molecules. The reactions possible would then be

$$\text{triosephosphate} + 2\text{MHb} = \text{phosphoglycerate} + 2\text{Hb}, \qquad (2)$$

Table 1. *The reduction of methaemoglobin in nitrite-treated erythrocytes from normal persons and from cases of idiopathic methaemoglobinaemia*

(Each manometer contained 2·0 ml. cell suspension, total pigment 15·6 g./100 ml. Cells suspended in 0·9% (w/v) NaCl made 0·01 M with phosphate buffer pH 7·4. Substrates added from Keilin tubes at the end of the equilibration period. Reaction period 4 hr. at 37°, gas phase 20% (v/v) CO in N_2. KOH papers in insets. Final concentrations, glucose 0·01 M, lactate 0·02 M.)

	MHb reduced (μmol.)			Pyruvate (μmol.)				Ratio Net pyruvate / Net MHb	
Normal bloods	Cells A	Cells + glucose B	Cells + lactate C	Cells initial D	Cells final E	Cells + glucose F	Cells + lactate G	Cells + glucose H	Cells + lactate I
1	1·0	3·9	3·9	0·5	3·6	2·4	5·1	0·49	0·52
2	0·9	3·0	3·5	0·3	2·1	1·8	3·3	0·50	0·46
3	0·8	3·6	4·1	0·3	3·5	2·0	5·5	0·47	0·49
4	1·0	3·2	—	0·4	2·9	2·0	—	0·50	—
5	1·2	2·7	3·0	0·5	2·4	1·8	3·4	0·48	0·55
6	2·0	6·0	6·6	0·5	4·4	3·8	6·8	0·53	0·52
Cases									
1	0·7	1·6	0·8	0·2	3·3	1·0	3·5	—	—
2	0·5	0·8	0·7	1·0	4·0	0·9	4·0	—	—
3	0·7	0·8	0·7	0·4	2·8	0·4	2·9	—	—
4	0·4	0·6	0·7	0·2	3·2	0·5	3·4	—	—
5	1·0	1·7	1·7	—	—	—	—	—	—
6	1·2	1·8	1·7	—	—	—	—	—	—

or glycolysis to lactate may be completed and reaction (1) follow. These reactions require coenzyme I. It is unfortunately impossible to test these reactions directly by trying out the intermediates postulated since the envelope of the erythrocyte is impermeable to the phosphorylated compounds, and the reduction of MHb effectively ceases on haemolysis.

Some indirect evidence is however available that reactions (1) and (2) are principally concerned.

(*a*) In glycolysis the oxidation of triosephosphate is coupled with the reduction of pyruvate so that the latter part of the scheme may be written

$$\text{triosephosphate} \xrightarrow{\text{oxidation}} \text{phosphoglycerate}$$

$$\text{lactate} \xleftarrow{\text{reduction}} \text{pyruvate}$$

Owing to the coupling of triosephosphate and pyruvate in the oxido-reduction, there can be no increase in the sum of the phosphoglycerate and pyruvate unless either triosephosphate or lactate is oxidized by some reaction outside the scheme. If the reduction of MHb takes place at the expense of either triosephosphate or lactate, then an increase in phosphoglycerate or pyruvate will occur. The figures in Table 1, col. H, i.e. cols. (F–D)/B, show that an amount of pyruvate equivalent to the amount of MHb reduced is formed in normal erythrocytes during the reduction of MHb by glucose. Net values, however, cannot be obtained by comparison with controls without substrate, because red cells contain a large amount of phosphoglycerate (Jost, 1927), which breaks down in the absence of glucose to give pyruvate (Table 1, cols. D, E). The cells from the cases of methaemoglobinaemia may be used as controls, since in them little reduction of MHb is taking place, and no accumulation of pyruvate occurs in the presence of glucose, while in the absence of glucose, pyruvate is formed in an amount similar to that found in normal cells (Table 1, cols. D, F, and D, E).

(*b*) In the presence of fluoride, the breakdown of phosphoglycerate is inhibited (Lohmann & Meyerhof, 1934), so that reduction of MHb by means of reaction (2) would be accompanied by the formation of an equivalent amount of phosphoglycerate. It has been found that reduction of MHb in the presence of glucose is not inhibited by 0·01 M-NaF, and that in its presence phosphoglycerate accumulates to a significant extent only when MHb is being reduced (Table 2). In the estimation of phosphoglycerate by the method of Rapoport (1936) it was found that with blood there was a tendency to give a greyish brown colour as compared with the blue of the standard, probably due to charring of organic material during heating with H_2SO_4 (Meyerhof & Schulz, 1938). For this reason, and also because of the large number of subtractions needed to obtain net values for comparison with the amounts of MHb reduced, close stoichiometric equivalence was not obtained, but the figures show that both MHb and glucose are required for substantial formation of phosphoglycerate.

Table 2. *The formation of phosphoglycerate in normal erythrocytes during the reduction of methaemoglobin in the presence of* 0·01 M-NaF

(Conditions as in Table 1, except that each manometer contained, in addition, 0·01 M-NaF. Incubation for 3 hr., except for the manometer containing methylene blue, which was incubated for 1 hr. only.)

	Phosphoglycerate (μmol.)		
	Washed cells	Nitrite-treated cells	MHb reduced (μmol.)
Initial	2·2	2·2	—
Final	2·8	3·2	2·5
Final + glucose	3·4	5·9	4·7
Final + glucose + 5×10^{-5} M-methylene blue	—	6·1	12·0

(c) Monoiodoacetate is known to inhibit the triosephosphoric enzyme. If MHb reduction depends on the oxidation of triosephosphate, it would be expected that iodoacetate would have an inhibitory effect, and, as shown in Table 3, it has been found

Table 3. *The effect of iodoacetate on the reduction of methaemoglobin in normal erythrocytes in the presence of glucose and lactate*

(Conditions as for Table 1.)

	MHb reduced (μmol.)			
	Gross		Net	
	Control	Iodoacetate 0·002 M	Control	Iodoacetate 0·002 M
Cells	0·8	0·8	—	—
Cells + glucose	3·5	1·0	2·7	0·2
Cells + lactate	4·0	4·0	3·2	3·2

that small concentrations of iodoacetate produce a 90% inhibition of MHb reduction. This effect is not due to a non-specific inhibitory action of iodoacetate, since reduction of MHb by lactate is unaffected.

Reduction of methaemoglobin by glucose and lactate in cells from cases of idiopathic methaemoglobinaemia

It has been found (Table 1, cols. A, B, C; Fig. 1) that there is relatively little tendency for MHb to disappear from the red cells of cases of idiopathic methaemoglobinaemia either in the presence or absence of glucose or lactate, and that there is correspondingly little formation of pyruvate during the experimental period (Table 1, cols. D, F and E, G). As the work on normal cells which has just been described had suggested that MHb reduction under these conditions depends chiefly on the triosephosphoric and lactic enzymes, the cells from the

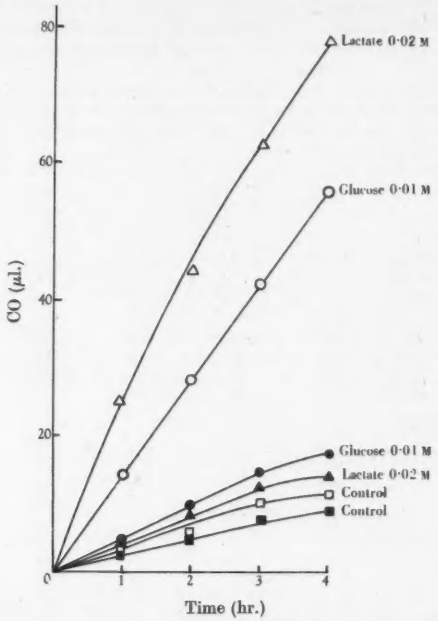

Fig. 1. Reduction of MHb by erythrocytes from a normal person and from a case of idiopathic methaemoglobinaemia. The ordinate gives the amount of MHb reduced, expressed in μl. CO absorbed by 2·0 ml. of a cell suspension containing 15·6 g./100 ml. total pigments, incubated at 37°. The clear symbols refer to the normal, the shaded symbols to the case. The conditions are the same as in Table 1.

Table 4. *The disappearance of pyruvate from normal erythrocytes and from erythrocytes from cases of idiopathic methaemoglobinaemia*

(Values given are μmol. pyruvate present/vessel. Each contained 2·0 ml. cell suspension in 0·9% (w/v) NaCl containing 0·01 M-phosphate buffer, pH 7·4. Total pigment 15·6 g./100 ml., 0·1 ml. 0·5 M-NaF, 0·2 ml. 0·029 M-Na pyruvate, and 0·2 ml. 0·1 M-glucose, or 0·2 ml. 0·9% (w/v) NaCl. Incubation at 37° in air for 1 hr.)

	Pyruvate (μmol.)		
Normals	Initial	Final	Final + glucose
1	6·8	6·4	2·4
2	6·8	6·3	2·0
3	6·8	6·4	3·0
4	6·8	6·5	2·0
5	6·8	5·8	3·0
Cases			
1	6·8	6·3	2·0
2	6·8	6·4	2·8
4	6·8	6·2	2·6

cases were investigated for a possible deficiency of these enzymes. To test this point, the disappearance of pyruvate in the presence of glucose and NaF was measured in normal and pathological cells. This reaction (Dische, 1937) involves both the triosephosphoric and lactic enzymes and diphosphopyridine nucleotide (DPN). The results given in Table 4 show that there is no significant difference, in this respect, between the normal and pathological cells. The cells from the cases are therefore not deficient in these enzymes and DPN (Fig. 8 A, 1–4).

The effect of methylene blue on the reduction of methaemoglobin by glucose and lactate

The finding of active triosephosphoric and lactic enzymes in the cells from cases of idiopathic methaemoglobinaemia, suggested that the failure of these cells to reduce MHb might be due to a deficiency of some oxidation-reduction carrier intervening between the pyridine nucleotides and MHb. In view of the effect of methylene blue in accelerating the reduction of MHb *in vivo* (Cox & Wendel, 1942), its action *in vitro* was examined. It was found, using normal cells (Table 5), that small amounts of the dye accelerated the reduction of MHb by glucose more than ten times. The rate of reduction by lactate was approximately doubled. Dewan & Green (1938) have shown that the reduction of methylene blue by reduced DPN is catalyzed by 'coenzyme factor', while Corran, Green & Straub (1939) have shown that 'coenzyme factor' is probably identical with the flavoprotein isolated by Straub (1939) from heart muscle. In the light of these findings it is probable that methylene blue acts as a carrier between this or a similar flavoprotein and MHb.

Table 5. *Reduction of methaemoglobin in the presence of* 5×10^{-5} M-*methylene blue by cells from cases of idiopathic methaemoglobinaemia and by cells from normal subjects*

(The figures show the amount of MHb reduced/hour. In comparing with Table 1, it should be noted that the figures there are for a period of 4 hr. The conditions are as for Table 1, except that each manometer contained 5×10^{-5} M-methylene blue in addition.)

	MHb reduced (μmol./hr.)		
Normals	Cells	Cells + 0·01 M-glucose	Cells + 0·02 M-lactate
1	0·8	8·2	3·0
2	0·9	13·0	—
3	1·1	16·2	3·9
4	1·3	—	4·5
5	1·8	9·6	6·1
Cases			
1	0·7	12·7	1·0
2	0·4	11·8	0·6
3	0·3	10·5	0·5
4	0·4	12·2	0·8
5	0·8	15·7	1·0
6	0·5	19·2	0·8

The results with the pathological cells are in striking contrast to the results with normal cells (Table 5). In the abnormal cells, the rate of reduction in the presence of methylene blue and glucose is accelerated in a manner similar to that found in normal cells. With lactate, however (Table 5; Fig. 2), the rate of MHb reduction is scarcely changed on the addition of methylene blue. This finding is notably different from that in normal blood, where the rate of reduction with lactate is substantially accelerated.

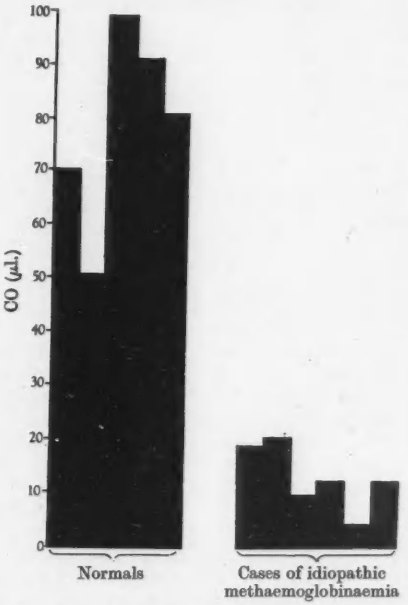

Fig. 2. MHb reduction by erythrocytes from normal persons and from cases of idiopathic methaemoglobinaemia in the presence of lactate and methylene blue. Each manometer contained 2·0 ml. nitrite-treated cell suspension (total pigment 15·6 g./100 ml.), 0·02 M-Na lactate and 5×10^{-5} M-methylene blue. The results are corrected for reduction of MHb taking place in controls without substrate. Temp. 37°, gas phase 20% (v/v) CO in N_2, reaction period 1 hr.

The pathway of the reaction in the presence of glucose and methylene blue is uncertain. The following evidence is available.

(*a*) The reaction does not appear to proceed via the triosephosphoric or lactic enzymes, since the amount of pyruvate accumulating is far below that

which would be expected if lactate were the ultimate reductant of MHb (Table 6), while the amount of phosphoglycerate accumulating in the presence of fluoride is similarly too small to account for the methaemoglobin reduced (Table 2).

Table 6. *The relation between methaemoglobin reduced and pyruvate formed in the presence of glucose and methylene blue*

(Conditions as in Table 1, except that each manometer contained 5×10^{-5} M-methylene blue. The figures for MHb are gross quantities reduced, the figures for expected pyruvate are calculated on the assumption that 1 mol. pyruvate corresponds to 2 mol. MHb. Incubation 4 hr.)

Normals	MHb reduced (μmol.)	Pyruvate formed (A) (μmol.)	Pyruvate expected (B) (μmol.)	100 A/B
1	9·4	1·09	4·7	23
2	7·5	0·80	3·8	21
3	10·6	1·28	5·3	24
4	6·4	1·05	3·2	32
5	12·6	1·46	5·3	23
Cases				
1	7·6	0·60	3·8	16
2	17·0	1·72	8·5	20
3	4·8	0·44	2·4	18
4	5·5	0·87	2·7	32

(*b*) Phosphorylation of glucose probably precedes the reduction of MHb, since arsenate, which interferes with phosphate transfer in the oxidation of triosephosphate (Needham & Pillai, 1937), produces a marked inhibition of MHb reduction. Oxalate, which is believed to inhibit the transfer of phosphate from phosphopyruvate (Lohmann & Meyerhof, 1934), produces less inhibition than arsenate, but the effect of the two together is more than additive. An experiment of this type is summarized in Fig. 3.

(*c*) When minimal quantities of glucose are present, the amount of MHb reduced/mol. glucose added exceeds the theoretical maximum possible, if oxidation of glucose were taking place after splitting of the sugar molecule to two 3-carbon compounds. If triosephosphate or lactate were the substrates concerned, the maximum possible ratio would be 4 mol. MHb/mol. glucose. In the presence of fluoride or oxalate, it has been found that this ratio may be substantially exceeded. An experiment of this kind is shown in Fig. 4. It should be noted that ratios of this kind represent minimum values, since any loss of glucose by side reactions such as glycolysis would tend to depress them.

The marked difference between normal and pathological bloods in the presence of glucose only, and the similarity of their behaviour in the presence of glucose and methylene blue, suggests that the addition of methylene blue virtually opens up a new

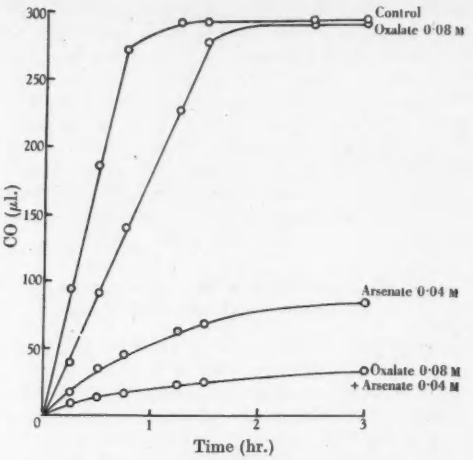

Fig. 3. The effect of arsenate and oxalate on the reduction of MHb in nitrite-treated erythrocytes in the presence of glucose and methylene blue. Each manometer contained 2·0 ml. cell suspension (total pigments 15·6 g./100 ml.), 0·01 M-glucose and 5×10^{-5} M-methylene blue. The glucose was added after equilibration at 37° from Keilin tubes. Gas phase 20% (v/v) CO in N_2.

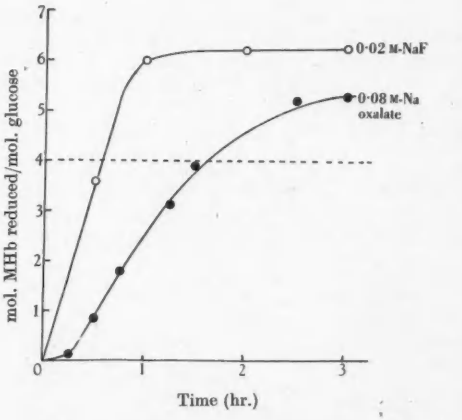

Fig. 4. The figure shows the amount of MHb reduced/mol. glucose added, after various periods of time. Each manometer contained 2·0 ml. nitrite-treated cell suspension in phosphate saline, 5×10^{-5} M-methylene blue, and 1 μmol. glucose which was added from a Keilin tube. The results are corrected for the reduction of MHb which took place in controls without added glucose. The theoretical maximum amount of MHb which could be reduced after splitting to two three-carbon compounds is indicated by the dotted line.

reaction pathway, rather than that it acts by accelerating a reaction which is already proceeding. These results may be accounted for by supposing that in the presence of methylene blue, stepwise oxidation of sugar occurs according to the scheme of Dickens (1938), who has shown that the necessary enzymes are present in the blood of the horse as well as in yeast (Fig. 8, col. B). In this step-wise breakdown of carbohydrate it would be possible for at least 8 mol. MHb to be reduced/mol. glucose used, and phosphorylation would precede the utilization of the sugar. Further, the enzymes in Dickens's (1938) scheme require triphosphopyridine nucleotide, as opposed to the enzymes taking part in the reduction of MHb in normal cells in the absence of methylene blue, which require DPN.

Coenzyme factor activity in normal red cells and in cells from cases of idiopathic methaemoglobinaemia

The finding that the cells from the cases of methaemoglobinaemia were unable to reduce MHb appreciably faster in the presence of lactate + methylene blue than in the presence of methylene blue alone (Table 5, Fig. 2) together with evidence that there are adequate amounts of the triosephosphoric and lactic enzymes in these cells (Table 4) suggested that the cells might be deficient in coenzyme factor activity. Measurements were therefore made of the rate of oxidation of triosephosphate in haemolysates prepared from normal blood and from the blood of the cases. It was found that under the conditions used (0·1 M-phosphate buffer, pH 7·4, 7×10^{-3} M-Na hexosediphosphate, 5×10^{-4} M-DPN, 2×10^{-4} M-methylene blue), the limiting factor governing the rate of oxidation of triosephosphate was the coenzyme factor when this was added in the form of a solution containing the Straub flavoprotein (Straub, 1939).

The results of assays of 'coenzyme factor' activity in twelve consecutive normal bloods and in the blood of the cases are given in Fig. 5, where the blackened portions correspond to the oxygen uptakes by the system specified. The total heights of the columns (shaded + unshaded) represent the uptakes in one-third of the time by the system + Straub flavoprotein where this uptake was determined.

The first three cases of methaemoglobinaemia examined gave results which are markedly lower than those obtained with normal blood. In the last two cases, however, the difference is much less striking. The assays of the first three cases were interpolated among assays of normal bloods, while the last two were carried out some time afterwards. The assays of the pathological bloods were in all cases repeated upon two separate blood samples taken at different times, with results which did not differ by more than 10%. After the assays of the last two cases had given higher results than for the other abnormal bloods, one of the earlier patients was recalled and the assay repeated. The result of this experiment confirmed the original low figure for this patient, so there does not appear to be any

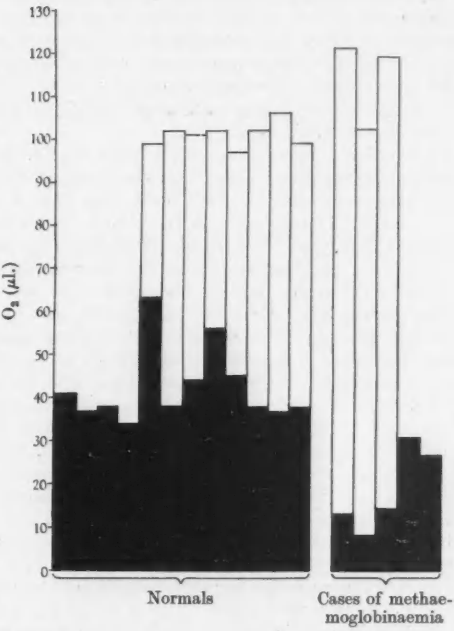

Fig. 5. The solid rectangles represent the amount of O_2 absorbed by 2·0 ml. of a haemolysate containing 5·6 g./100 ml. total pigment, in the presence of 0·1 M-phosphate buffer pH 7·4, 0·007 M-Na hexosediphosphate, 5×10^{-4} M-diphosphopyridine nucleotide, and 2×10^{-4} M-methylene blue. The heights of the whole columns (shaded + unshaded) represent the amount of O_2 absorbed by the same system in the presence of 0·4 ml. of a preparation of the Straub flavoprotein. The methylene blue was added from Keilin tubes. The figures for the shaded portions give the uptakes from 15 min. after the addition of the dye until 60 min. afterwards. The whole columns represent the uptakes in the first 15 min. after the addition of methylene blue. Incubation at 37° in air. In the last two cases examined the preparation of Straub flavoprotein had become relatively inactive. It produced a large increase in both cases, but the final figures were not considered reliable and so have been omitted.

doubt either of the low figures for the first three cases examined, or of the higher figures for the last two. It should be noted that the last two cases were much less severely affected than the others, having initial blood MHb levels of less than 2 g./100 ml. while the first three had initial MHb levels ranging from 5·1 to 7·8 g./100 ml. The last two cases, nos. 5 and 6 in Table 1, also showed a higher enzyme activity in the

intact cells. Taking the results as a whole, it would seem justifiable to conclude that a definite deficiency in the coenzyme factor activity of the blood of persons suffering from idiopathic methaemoglobinaemia has been demonstrated. The coenzyme factor which was tested in these experiments is coenzyme factor I reacting with DPN. The deficiency of this factor in the cells from the cases provides a satisfactory explanation of the failure of methylene blue to accelerate lactate oxidation in the intact pathological cells.

Correction required in coenzyme factor assays. In carrying out these experiments it was assumed that the rate of reaction between methylene blue and reduced DPN is negligible in the absence of flavoprotein, but later work showed that this is by no means true, and that the figures obtained required correction for the oxygen transferred by direct reaction between reduced DPN and methylene blue. As the size of this correction had an important bearing on the comparisons made between normal and abnormal bloods it was determined with some care. Reduced DPN was prepared by the method of Ohlmeyer (1938) and allowed to react with methylene blue in phosphate buffer in air at 37°. The reaction was followed manometrically. The results, plotting the logarithm of reduced DPN concentration against time for two different methylene blue concentrations, show that the reaction is apparently bimolecular (Fig. 6). This allows of the calculation of the correction for any required amounts of DPN and methylene blue.

(*a*) In the lactic enzyme system prepared from acetone powder of heart muscle extract, varying amounts of DPN were added in the presence of 0·065M-HCN, 0·08M-phosphate buffer pH 7·4, and 0·016M-Na lactate. The results of several experiments gave a mean value for the correction of 28 μl./hr.

(*b*) Experiments in which varying amounts of DPN were added to blood haemolysates showed that the addition of the amount required to give a final concentration of 6×10^{-5}M approximately doubled the oxygen uptake, while further additions up to a concentration of 10^{-3}M gave uptakes which increased slowly and gradually beyond this point. These results suggested that the enzyme system was almost effectively saturated at the lower concentration and that the slow increase on further addition of DPN was due to the direct reaction between reduced DPN and methylene blue. The correction derived from these experiments was 26 μl./hr. These independent methods of determining the correction lead to practically the same result, and the average value has been used in correcting the coenzyme factor assays.

It will be noted that in Fig. 5 the oxygen uptakes have been recorded over a period of 45 min. which began 15 min. after the addition of methylene blue from a Keilin tube. The readings in the initial period were disregarded. This was done for two reasons, namely that in the controls without substrate, a small amount of MHb is formed by oxidation by the

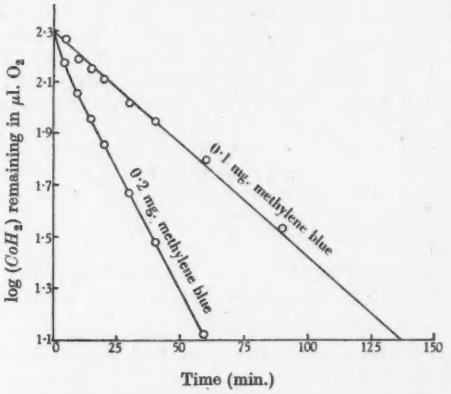

Fig. 6. Oxidation of reduced diphosphopyridine nucleotide by molecular O_2 in the presence of methylene blue. Each manometer contained 5·9 mg. reduced diphosphopyridine nucleotide (CoH_2), in 0·1M-phosphate buffer, pH 7·4. The quantity of methylene blue shown on the graph was added from a Keilin tube after temperature equilibration. Temp. 37°, gas phase air. The ordinate shows the amount of reduced diphosphopyridine nucleotide present at any time expressed as the log. of the equivalent amount of O_2. As H_2O_2 is the end product under the conditions used, the figures are twice those which would be observed in the presence of catalase.

methylene blue, and that when the blood from the cases was used, which already contained a large amount of MHb, rapid reduction occurred as soon as the methylene blue was added and continued until an equilibrium amount of MHb was present. As the transfer of 1 electron will lead to the uptake of 1 mol. O_2 when applied to the reduction of MHb, whereas 4 electrons must be transferred when H_2O is the end product, it is desirable to postpone the initial reading until reasonable time has been allowed for equilibrium with regard to MHb, and the reaction has settled down to a steady rate.

The oxidation of hexosemonophosphate in haemolysates

In view of the suggestion that the reduction of MHb in the presence of glucose + methylene blue may proceed by means of coenzyme II dehydrogenases, it was of interest to examine the oxidation of hexosemonophosphate in the blood of normal persons and in the blood of the cases. Owing to the difficulty of preparing coenzyme II, fully detailed

studies were not carried out, but it was found, as would be expected if this suggestion is correct, that the rate of oxidation of hexosemonophosphate was substantially similar in both normal and abnormal bloods (Fig. 7).

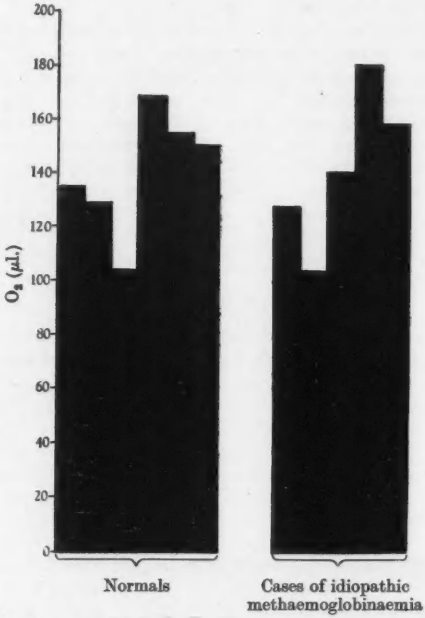

Fig. 7. Oxidation of hexosemonophosphate in haemolysates of normal blood and of blood from cases of idiopathic methaemoglobinaemia. Each manometer contained 2·0 ml. haemolysate (total pigment 5·6 g./100 ml.), 2×10^{-4} M-methylene blue, 5×10^{-4} M-diphosphopyridine nucleotide and 0·07 M-Na hexosemonophosphate. The methylene blue was added from Keilin tubes after temperature equilibration. The height of the columns represents the amount of O_2 absorbed in 1 hr. at 37° after the addition of the methylene blue. Gas phase air.

DISCUSSION

On the basis of the work which has been described, the following tentative explanation is offered for the occurrence of idiopathic methaemoglobinaemia, and of the normal mechanism for the reduction of MHb in the blood.

(*a*) MHb reduction requires coenzyme I dehydrogenases, particularly the lactic and triosephosphoric dehydrogenases. Associated with these is coenzyme factor I. It is assumed that coenzyme II dehydrogenases do not play any very significant part in the reduction of MHb under normal circumstances, probably because coenzyme factor II does not react with MHb.

(*b*) On the addition of methylene blue, which is capable of reacting rapidly with both coenzyme factors I and II and MHb, the reduction of MHb is greatly speeded up both by an acceleration of the existing pathway via coenzyme I dehydrogenases and the opening up of new pathways via coenzyme II dehydrogenases in Dickens's scheme. These reactions are presented schematically in Fig. 8.

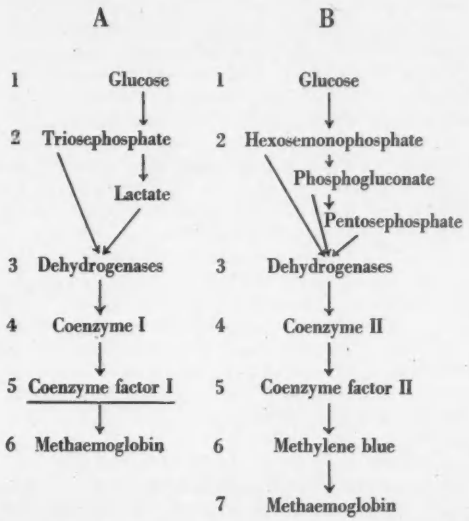

Fig. 8. Diagrammatic scheme of the reactions taking place in the reduction of MHb in erythrocytes. Column A reactions occurring in the absence of methylene blue; B in the presence of methylene blue. In cases of idiopathic methaemoglobinaemia where there is a deficiency of coenzyme factor I, the reactions in column A do not occur, and MHb reduction is very slow.

(*c*) MHb reduction fails to occur in the cases of idiopathic methaemoglobinaemia because of the deficiency of coenzyme factor I. The addition of methylene blue speeds up MHb reduction as with normal blood because of the presence of coenzyme II dehydrogenases and coenzyme factor II, together with glucose. In the presence of lactate, there is no marked effect on adding methylene blue because the lactic enzyme requires coenzyme I and coenzyme factor I which is present only in small amounts.

(*d*) MHb does not continue to increase in amount in the blood of persons affected with idiopathic methaemoglobinaemia to an unlimited extent, because, as the concentration of MHb rises, its rate of reaction with non-specific reducing agents in the blood will also rise and an equilibrium will be established. In this respect it is probable that ascorbic acid and glutathione are the most important

reducing agents in the blood. The results of Gibson (1943) suggest that ascorbic acid reacts directly with MHb and the dehydroascorbic acid formed is again reduced by glutathione. The action of large amounts of ascorbic acid in bringing about a new equilibrium with much smaller amounts of MHb in the blood of these cases (Lian *et al.* 1939; Deeny, Murdock & Rogan, 1943; Barcroft *et al.* 1945; Sievers & Ryon, 1946) is probably due to a mass action effect of this kind.

It should be pointed out that direct evidence is not available for all the assumptions and suggestions made in this scheme. It has, however, the merit of accounting for all the reactions observed in the abnormal cells by means of a single deficiency, which has been demonstrated with reasonable assurance, namely the deficiency of coenzyme factor I. The difficulties met with in the work have been of two kinds: first, it has not been possible to obtain blood samples from the patients as often and at the times which would have been desirable, because none of the patients lived in Belfast, but had to travel for considerable distances to the laboratory; and secondly, it has been impossible to put the theories about the intermediates involved in the various reactions to a direct experimental test, since the red cells are impermeable to most of the substrates involved, and laking the cells stops the reduction of MHb.

There seems to be no doubt, however, that idiopathic methaemoglobinaemia belongs to the group of diseases due to an inborn error of metabolism and should be classified along with alcaptonuria and cystinuria rather than under enterogenous cyanosis. Of the alternative explanations possible for the accumulation of MHb in the blood of these cases, impermeability of the erythrocytes to glucose may be dismissed, since glucose is rapidly oxidized by the cells in the presence of methylene blue, while a rapid rate of formation of MHb also seems to be improbable, otherwise reducing systems would lead to the disappearance of the pigment from the shed blood as occurs in drug methaemoglobinaemias where the cells are normal. In this connexion it should be noted that Sievers & Ryon (1946) examined the blood of a case with great care for MHb-forming agents without finding any evidence of their presence.

The results bearing on the mechanism of MHb reduction in normal red cells are of considerable interest, since it is probable that MHb is constantly being formed in the blood of normal persons (Peters & Van Slyke, 1931). The low level which is present in normal blood, about 1 % of total pigment (Gibson & Harrison, 1945), would presumably represent the amount required to give equilibrium between the rates of formation and removal of the pigment. If there is such an equilibrium between formation and removal, the enzymatic mechanism described would account for the removal of MHb and the maintenance of the haemoglobin in a functional state in normal persons. Failure of any part of this enzyme system to function would lead to methaemoglobinaemia of the type exemplified by the patients described in this paper.

SUMMARY

1. Methaemoglobin reduction in normal erythrocytes takes place through the oxidation of triosephosphate and lactate.

2. The system requires coenzyme factor I in addition to the enzymes and coenzyme I.

3. Coenzyme II dehydrogenases do not play a part in the normal mechanism of methaemoglobin reduction, but on the addition of methylene blue, they take the principal part in bringing about the great acceleration of methaemoglobin reduction which is observed.

4. Methaemoglobin present in the blood of persons suffering from idiopathic methaemoglobinaemia is not reduced with appreciable speed in the presence of glucose or lactate.

5. Blood from cases of idiopathic methaemoglobinaemia contains less coenzyme factor I activity than does normal blood.

6. It is suggested that, owing to the deficiency of coenzyme factor I in the blood of patients suffering from idiopathic methaemoglobinaemia, the enzymatic reducing activity is lower than in normal cells, leading to a displacement of the equilibrium Hb $\rightleftharpoons$ MHb to the right and the consequent accumulation of abnormal amounts of MHb in the blood.

The author wishes to thank Prof. D. C. Harrison for his interest in the work.

REFERENCES

Barcroft, H., Gibson, Q. H., Harrison, D. C. & McMurray, J. (1945). *Clin. Sci.* **5**, 145.

Clegg, J. W. & King, E. J. (1942). *Brit. med. J.* **2**, 329.

Corran, H. S., Green, D. E. & Straub, F. B. (1939). *Biochem. J.* **33**, 793.

Cox, W. W. & Wendel, W. B. (1942). *J. biol. Chem.* **143**, 331.

Deeny, J., Murdock, E. T. & Rogan, J. J. (1943). *Brit. med. J.* **1**, 721.

Dennig, A. (1900). *Dtsch. Arch. klin. Med.* **65**, 524.

Dewan, I. G. & Green, D. E. (1938). *Biochem. J.* **32**, 626.

Dickens, F. (1938). *Biochem. J.* **32**, 1626.

Dische, Z. (1937). *Enzymologia*, **1**, 288.

DuBois, K. P. & Potter, V. R. (1943). *J. biol. Chem.* **147**, 41.
Evelyn, K. A. & Malloy, H. T. (1938). *J. biol. Chem.* **126**, 655.
Gibson, Q. H. (1943). *Biochem. J.* **37**, 615.
Gibson, Q. H. & Harrison, D. C. (1945). *Biochem. J.* **39**, 490.
Green, D. E. & Brosteaux, J. (1936). *Biochem. J.* **30**, 1489.
Green, D. E. & Williamson, S. (1940). *J. biol. Chem.* **135**, 345.
Jost, H. (1927). *Hoppe-Seyl. Z.* **165**, 171.
Lian, C., Frumusan, P. & Sassier, M. (1939). *Bull. Soc. méd. Hôp. Paris*, **55**, 1194.
Lohmann, K. & Meyerhof, O. (1934). *Biochem. Z.* **273**, 60.
Long, C. (1942). *Biochem. J.* **36**, 807.
Meyerhof, O. & Schulz, W. (1938). *Biochem. Z.* **297**, 60.
Morgan, W. J. T. & Robison, R. (1930). *Biochem. J.* **24**, 119.
Needham, D. M. & Pillai, R. K. (1937). *Biochem. J.* **31**, 1837.
Neuberg, C. & Vercellone, A. (1935). *Biochem. Z.* **280**, 161.
Ohlmeyer, P. (1938). *Biochem. Z.* **297**, 66.
Paul, W. D. & Kemp, C. R. (1944). *Proc. Soc. exp. Biol., N.Y.*, **56**, 55.
Peters, R. A. (1938). *Biochem. J.* **32**, 2031.
Peters, J. P. & Van Slyke, D. D. (1931). *Quantitative Clinical Chemistry*, Interpretations, p. 631. London: Baillière, Tindall and Cox.
Rapoport, S. (1936). *Biochem. Z.* **289**, 406.
Robison, R. (1922). *Biochem. J.* **16**, 809.
Sievers, R. F. & Ryon, J. B. (1946). *Arch. intern. Med.* **76**, 299.
Stadie, W. C. (1921). *J. exp. Med.* **33**, 627.
Straub, F. B. (1939). *Biochem. J.* **33**, 787.
Warburg, O. & Christian, W. (1931). *Biochem. Z.* **242**, 174.
Warburg, O., Christian, W. & Griese, A. (1935). *Biochem. Z.* **282**, 157.
Warburg, O., Kubowitz, F. & Christian, W. (1930). *Biochem. Z.* **227**, 245.

Studies on Glyoxalase

1. A NEW FACTOR

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The experiments to be described took origin from a personal observation by one of us (E.J.M.). He found that the enzyme glyoxalase was well adsorbed on calcium phosphate gel at pH 5–6, the filtrate showing no activity at all in the presence of methylglyoxal and glutathione. When, however, a proportionate amount of the filtrate was added to the eluate from the calcium phosphate the activity of this was largely increased.

EXPERIMENTAL

General methods

The method was that used in our previous paper (Hopkins & Morgan, 1945), namely, the determination of the rate of CO_2 evolution from $NaHCO_3$ solution, due to lactic acid production from methylglyoxal. We employed Barcroft differential manometers provided with standardized flasks. Each flask contained a measured quantity of the enzyme solution or other preparations, 0·6 ml. of 0·2M-$NaHCO_3$, with 2 mg. of glutathione (GSH) in 0·3 ml. water in a Keilin cup. To one flask 2·1 mg. of methylglyoxal were added, and the total contents of each flask made up to 3 ml. with distilled water (any variation of these quantities is given in the text). The apparatus was filled with a gas mixture of 95 vol. N_2 + 5 vol. CO_2, the GSH tipped in and the manometer shaken in a bath at 25°. The stopcocks were left open for 4 min. and then closed.

The majority of our experiments were carried out with ox heart, as this was the most convenient tissue to obtain fresh in sufficient quantities. Acetone preparations of the hearts were prepared. In making these, owing to the scarcity of acetone, smaller quantities were used than usual. The hearts freed from fat were minced, and extracted at 0° with three times their weight of acetone for 30 min., filtered, and extracted a second time for 15 min., using half the previous volume of acetone. The extracted heart was filtered off and dried in large vacuum desiccators. It was then ground to a fine powder in a mill. This acetone heart powder if kept dry keeps its activity for several months.

Separation of the new factor from the enzyme

The following separation was repeated several times; the results are shown in Table 1.

1st stage. 80 g. of acetone heart powder were extracted by stirring with 400 ml. of distilled water for 1 hr. and then centrifuged. The supernatant, which was highly pigmented, measured 234 ml. Of this, 0·3 ml. (dry wt. 9·90 mg.) gave 303·2 μl. CO_2 in 20 min.

2nd stage. Fractional precipitation with ammonium sulphate. To 220 ml. of the supernatant, solid ammonium sulphate (68·64 g.) was slowly added to give 50 % saturation. After standing 2 hr. the precipitate was centrifuged off and discarded.

The filtered, clear supernatant measured 234 ml. The ammonium sulphate concentration was then raised to 70% saturation by adding the calculated amount of the solid (31·6 g.). After standing overnight in the refrigerator, the precipitate, which contained both the factor and the enzyme, was filtered off on two ordinary funnels, and given one good wash by filling up the papers with 70% saturated ammonium sulphate. The precipitate was scraped off the paper, dissolved in about 50 ml. of distilled water and dialyzed in cellophan tubing against distilled water, with frequent changes, for 18–24 hr. An inactive precipitate was centrifuged off and the supernatant diluted to 110 ml. (i.e. half the volume originally taken). The pH of this solution was approximately 5·4 and needed no adjustment for the next stage. 0·3 ml. (dry wt. 5·25 mg.) gave 302·5 μl. CO_2 in 20 min.

3rd stage. Adsorption of the enzyme. 100 ml. of the above solution were used for this stage. The enzyme was completely adsorbed on calcium phosphate gel suspension (30 mg. dry wt./ml.) by adding four successive lots of 10 ml., allowing each to stand 15 min. with an occasional stir, and then each time centrifuging off the gel. The supernatant contained the factor (see Table 1). 0·42 ml. (dry wt. 3·24 mg.), equivalent to 0·3 ml. of the 100 ml. taken for stage 3, gave 21·0 μl. CO_2 in 20 min.

Elution of the enzyme. The four lots of calcium phosphate gel containing the enzyme were mixed, and washed well by stirring with 200 ml. of distilled water to which was added 10 ml. of Sørensen's phosphate buffer pH 5·4. This washing was repeated three times. The enzyme was then eluted from the calcium phosphate by two successive extractions with phosphate buffer pH 7·8 containing 10% (w/v) ammonium sulphate, 50 and 20 ml. respectively being added. The two eluates were mixed, dialyzed for 24 hr., made up to 100 ml. and a very small inactive precipitate filtered off. Of the enzyme solution 0·3 ml. (dry wt. 1·5 mg.) gave 123·3 μl. CO_2 in 20 min.; 0·3 ml. enzyme + 0·42 ml. of factor gave 279·0 μl. CO_2 in 20 min. Other results are shown in Table 1. Preparations 1 and 2 were made from separate supplies of hearts.

It will be seen from the results (Table 1) that the presence of the factor has in each case more than doubled the velocity of action of the enzyme alone, the average increase being 141·7%. In each case the enzyme and factor are equivalent to the same weight of heart powder.

Table 1. *Evidence for the new factor*

Preparation	Acid production from methylglyoxal (μl. CO_2 evolved from $NaHCO_3$ in 20 min.) By 0·3 ml. enzyme	By 0·42 ml. factor	By 0·3 ml. enzyme + 0·42 ml. factor
1	82·2	21·0	183·0
1	75·0	22·0	216·0
1	74·0	18·0	228·0
1	96·0	21·0	186·0
1	95·1	21·0	246·0
2	138·0	23·1	294·0
2	74·5	22·0	216·0
2	115·6	15·4	246·0
Mean	93·8	20·4*	226·8

* This small reading is not of enzymic origin, but due to some chemical action between GSH and methylglyoxal (Jowett & Quastel, 1933; Hopkins & Morgan, 1945).

Nature of the action of the factor

It is apparent from Fig. 1 that the effect of the factor is accelerative, maximal CO_2 liberation with enzyme + factor being attained in 20 min.; with enzyme alone in 140 min. The stopcocks were open in this experiment for 5 min., therefore the μl. of CO_2 evolved during this time, which we may take from the curves to be 60·0 and 25·27 respectively, must be added to the amount evolved (Fig. 1) affording totals for the enzyme + factor of 282·7 and for the enzyme alone of 282·0 μl.

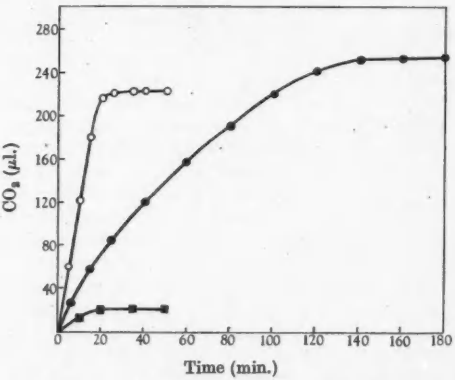

Fig. 1. Effect of factor on the action of glyoxalase. Curve ●—●, 0·3 ml. enzyme; ■—■, 0·42 ml. factor; ○—○, 0·3 ml. enzyme + 0·42 ml. factor. 1·0 mg. methylglyoxal and 2·0 mg. GSH in each. The dry weight of 0·3 ml. enzyme was 1·65 mg. and of 0·42 ml. factor 0·82 mg.

Further purification and properties of the factor

The factor was further purified by the following three additional stages.

4th stage. To 112 ml. of the supernatant (stage 3) 45·9 g. of ammonium sulphate were slowly added giving a final concentration of 63% saturation. After standing 2 hr. the precipitate was filtered off

and washed with 63 % saturated ammonium sulphate. The precipitate was scraped off the filter paper, dissolved in distilled water, dialyzed 18 hr. and made up to 80 ml. (this is the same dilution as the finish of stage 2). Of this solution 0·3 ml. (dry wt. 0·82 mg.) + 0·3 ml. enzyme gave 267·0 μl. CO_2 in 20 min.

5th stage. The solution was adjusted to pH 7·3 with sodium bicarbonate and heated in a water bath to 55° for 1 min. The solution was quickly cooled and the coagulum centrifuged off. Of the supernatant 0·3 ml. (dry wt. 0·38 mg.) + 0·3 ml. enzyme gave 237·0 μl. CO_2 in 20 min. It will be seen that there was some loss in activity of this particular preparation by heating. In all subsequent preparations there was no loss by heating to 55°.

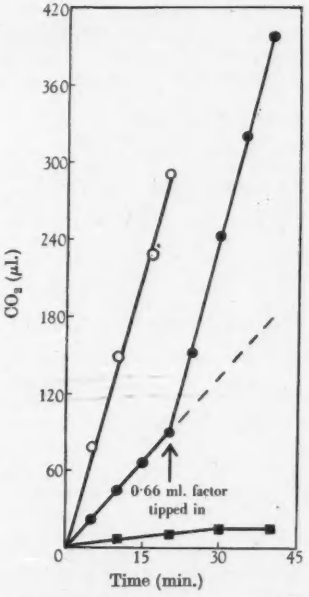

Fig. 2. Promptness of action of the factor. Curve ○—○, 0·3 ml. enzyme + 0·66 ml. factor; ●—●, 0·3 ml. enzyme, with 0·66 ml. factor tipped in at arrow; ■—■, 0·66 ml. factor, 2·0 mg. GSH and 2·1 mg. methylglyoxal in each. The dry weight of 0·3 ml. enzyme was 1·0 mg., and of 0·66 ml. factor 0·308 mg.

6th stage. 50 ml. were adjusted to pH 5·4 with dilute acetic acid and 5 ml. of calcium phosphate gel added (in later preparations 20 ml. were used). After standing 15 min. the calcium phosphate was centrifuged off and discarded. 0·33 ml. (dry wt. 0·154 mg.) + 0·3 ml. enzyme gave 201·0 μl. CO_2 in 20 min. For the dry weight of the 5th and 6th stage, measured portions were dialyzed. At this stage the solution was crystal clear. The factor can be concentrated by precipitating with ammonium sulphate (final concentration 63 % saturated) taking the precipitate up in the minimal quantity of distilled water and dialyzing until free from ammonium sulphate. Working on a much larger scale the solutions were kept more concentrated after the 2nd stage. One large scale attempt to crystallize the factor from ammonium sulphate failed, the activity decreasing, probably due to the acidity of the ammonium sulphate. In the following experiments we used the purified factor.

Action of factor immediate. The factor prepared as above exerts its full effect immediately it is added to the system, i.e. without lapse of time for any interaction with the reactants; this is shown in Fig. 2.

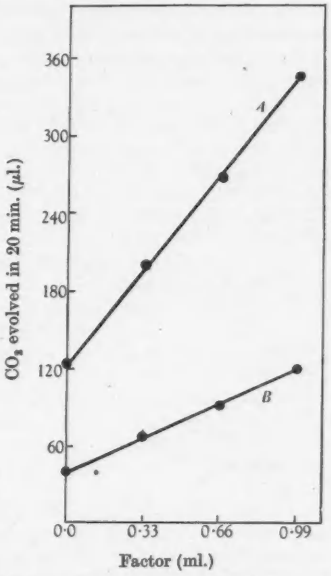

Fig. 3. Effects of varying quantities of the factor on glyoxalase action. 0·3 ml. of enzyme in each Barcroft flask, with varying amounts of factor. Dry weights the same as Fig. 2. Curve *A* with 2·0 mg. GSH, and curve *B* with 0·25 mg. GSH; 2·1 mg. methylglyoxal in each.

Variation of velocity of reaction with factor concentration. Fig. 3 shows the effect on the velocity of reaction of increasing the concentration of the factor with a constant volume of enzyme.

Dependence of action by the factor upon presence of GSH. Three Barcrofts were set up as follows: *A*, one flask with 0·3 ml. enzyme (1·0 mg.) + 0·6 ml. factor (0·2 mg.) + GSH but no methylglyoxal; the other flask with 2·4 ml. water + 0·6 ml. bicarbonate; *B*, with enzyme + factor + methylglyoxal but no GSH;

C, with enzyme + factor + methylglyoxal + GSH. There was no CO_2 evolution in *A* and *B*, whilst in *C*, 207·0 μl.CO_2 were evolved in 20 min.

Experiments with phenylglyoxal as the substrate

Using preparations of glyoxalase as ordinarily made, the velocity of the reaction was very much slower with phenylglyoxal than with methylglyoxal. Equimolar quantities of the substrates were used. The results are shown in Table 2. The enzyme preparations were made by grinding 10 g. of tissue with 30 ml. of water in a mortar for 30 min., using a little sand, and then centrifuging. The supernatant was dialyzed for 24 hr., made up to 50 ml. and filtered. 0·3 ml. of the preparations were used in the Barcroft.

Relation of the factor to phenylglyoxal. In Table 3 it will be seen that when the enzyme is separated from the factor, the former acts with about equal velocity on methyl- and phenylglyoxal. When, however, the factor is added to the enzyme, it greatly increases the velocity of reaction with methylglyoxal whereas in the case of phenylglyoxal it produces no effect.

Table 2. *Methyl and phenylglyoxals as substrates*

Tissue	Time (min.)	CO_2 evolved from $NaHCO_3$ (μl.) With 4·0 mg. phenylglyoxal	With 2·1 mg. methylglyoxal
Ox heart	20	77·1	135·0
Ox liver	10	90·0	287·8
Rat liver	10	72·0	225·0
Rat erythrocytes	10	108·0	237·0
Sheep liver	10	90·0	315·0
Pig liver	20	180·0	309·0
Acetone ox heart	20	99·0	300·0
Acetone yeast	20	138·0	223·6

It will be seen from the following table (Table 4) that phenylglyoxal combines with the factor. On adding phenyl and methylglyoxal in equimolar proportions to the enzyme + factor (Exp. G) there was

Table 3. *Differential effect of the factor on reaction with methylglyoxal*

Substrate	CO_2 (μl.) evolved in 20 min. from $NaHCO_3$, with 0·3 ml. enzyme	0·3 ml. enzyme + 0·42 ml. factor
(*a*) Methylglyoxal, 2·1 mg.	96·2	186·0
Phenylglyoxal, 4·0 mg.	102·0	96·0
	0·3 ml. enzyme (1·5 mg.)	0·3 ml. enzyme + 0·2 ml. factor (0·2 mg.)
(*b*) Methylglyoxal, 2·1 mg.	79·6	156·0
Phenylglyoxal, 2·0 mg.	74·2	66·0
	0·3 ml. enzyme (1·0 mg.)	0·3 ml. enzyme + 0·66 ml. factor (0·308 mg.)
(*c*) Methylglyoxal, 2·1 mg.	123·3	267·0
Phenylglyoxal, 4·0 mg.	120·0	118·2
	0·3 ml. enzyme (0·9 mg.)	0·3 ml. enzyme + 0·6 ml. factor (0·6 mg.)
(*d*)* Methylglyoxal, 2·1 mg.	54·0	163·6
Phenylglyoxal, 2·0 mg.	41·0	45·0

* 0·5 mg. GSH.

Table 4. *Combination of the factor with phenylglyoxal*

(The reactants were measured into the Barcroft flasks in the following order: water, bicarbonate, 0·3 ml. enzyme, 0·6 ml. factor, methylglyoxal, phenylglyoxal, and 0·5 mg. GSH tipped in. Dry weights in experiments A–I: 0·3 ml. enzyme, 0·99 mg.; 0·6 ml. factor, 0·6 mg. J–L, and M and N, contained different preparations of the enzyme and factor. The final concentrations of the substrates are given in brackets.)

Exp.	Reaction mixture	CO_2 evolved in 20 min. (μl.)
A	Enzyme + 2·1 mg. methylglyoxal (0·01 M)	41·1
B	+ factor + 2·1 mg. methylglyoxal (0·01 M)	105·0
C	+ 4·0 mg. phenylglyoxal (0·01 M)	41·1
D	+ factor + 4·0 mg. phenylglyoxal (0·01 M)	42·0
E	+ 2·0 mg. phenylglyoxal (0·005 M)	42·0
F	+ 1·0 mg. phenylglyoxal (0·0025 M)	36·0
G	+ 4·0 mg. phenylglyoxal (0·01 M) + 2·1 mg. methylglyoxal (0·01 M)	42·0
H	+ 2·0 mg. phenylglyoxal (0·005 M) + 2·1 mg. methylglyoxal (0·01 M)	66·0
I	+ 1·0 mg. phenylglyoxal (0·0025 M) + 2·1 mg. methylglyoxal (0·01 M)	74·5
J	+ 2·1 mg. methylglyoxal	123·0
K	+ 2·0 mg. phenylglyoxal	56·5
L	+ 2·0 mg. phenylglyoxal dialyzed 24 hr. + 2·1 mg. methylglyoxal	111·0
M	+ 2·1 mg. methylglyoxal	156·0
N	+ 2·1 mg. methylglyoxal + 1·125 mg. mandelic acid	154·0

no increase in velocity; lowering the amount of phenylglyoxal so that the methylglyoxal was in the proportion of 2 to 1 (Exp. H) gave an increase of 24 μl. CO_2 evolved, and a 4 to 1 ratio (Exp. I) gave 32·5 μl. This shows that phenylglyoxal has the greater affinity for the factor but the velocity of its enzymic decomposition is not increased as is the case with methylglyoxal. That the phenylglyoxal can be dialyzed away from the factor is shown by Exps. J, K and L. Mandelic acid does not inhibit in concentrations that would be formed during the experiments (Exps. M and N).

The factor is protein in nature, or closely associated with a protein. The solutions give good biuret and ninhydrin reactions, and positive colour tests for tyrosine, tryptophan, arginine, histidine and cystine.

The purest preparation of the factor we have so far prepared, when used in a quantity of 0·1 mg. with 1·0 mg. of enzyme, increased the velocity of reaction by 66·6%. The activity was completely destroyed by heating at 70° for 3 min.

DISCUSSION

It will be seen from the results of the experiments described above that when methyl- and phenylglyoxal are added to a preparation of glyoxalase as ordinarily made from a tissue, the velocity of the reaction with the former is often more than three times that with the latter substrate (Table 2).

Lohmann (1932), in his experiments showing that glutathione was the coenzyme for glyoxalase, obtained this effect with either (he did not state which) rat or rabbit liver.

There seems to be no doubt that this increased velocity, when methylglyoxal is the substrate, is due to the new factor which we have separated and purified. We have shown that when the enzyme and factor are separated, the velocity of the reaction with the enzyme alone is the same for both substrates. But when the factor is added to the enzyme it is only the methylglyoxal which shows increased velocity, the phenylglyoxal giving the same evolution of CO_2 as for the enzyme alone (Table 3). This is shown to be due to the fact that, although phenylglyoxal combines with the factor, such combination does not increase the velocity of reaction (Table 4). We have emphasized earlier (Fig. 1) that the essential effect of the presence of the factor is an accelerative one.

In respect of the separation of the enzyme from the factor, it was successful enough in that solutions of the latter show no trace of enzyme activity. It is possible that the partially purified preparations of the enzyme as described, might leave it in association with small quantities of the factor, and that these are responsible for, and essential to, the action of the enzyme on methylglyoxal. It seems to us that strong evidence against this is yielded by the identical velocity of reaction displayed when methyl- and phenylglyoxal react with our preparations of the enzyme. Since the factor has no effect in the case of phenylglyoxal, it would if present as an impurity secure a greater velocity for the methylglyoxal. Furthermore, as the factor is completely inhibited by phenylglyoxal (Table 4), any traces left in the enzyme solution would also be inhibited, therefore it follows that if the enzyme needed the factor for its activity, phenylglyoxal would not react to give an evolution of CO_2.

We hope that further work, which is in progress, may elucidate the role of the new factor.

SUMMARY

1. A preliminary purification of glyoxalase is described.

2. A new factor, present in tissues, is shown to accelerate the conversion of methylglyoxal to lactic acid.

3. Phenylglyoxal inhibits the factor.

4. The factor has been separated and purified.

REFERENCES

Hopkins, F. G. & Morgan, E. J. (1945). *Biochem. J.* **39**, 320.

Jowett, M. & Quastel, J. H. (1933). *Biochem. J.* **27**, 486.

Lohmann, K. (1932). *Biochem. Z.* **254**, 332.

Anti-histamine Effect of Sodium Salicylate and its Bearing upon the Skin-diffusing Activity of Hyaluronidase

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It was claimed by Guerra (1946 *a*, *b*) that the administration of sodium salicylate to rabbits in a dosage of 0·1 g./kg. body weight inhibited the skin-diffusing activity of hyaluronidase to a significant extent. Swyer (1948), on the other hand, failed to find any inhibitory effect of salicylates on the viscosity-reducing activity of hyaluronidase except when the concentration of the former reached such high levels that the accompanying reduction of pH itself caused inhibition of the enzyme action. In an attempt to account for this discrepancy between the *in vivo* and *in vitro* results, it was suggested that the hyaluronidase preparation used by Guerra might have contained histamine, or some substance with a similar action, which, by increasing the capillary permeability, would have enhanced the spreading effect of the hyaluronidase. The further suggestion was made that salicylate might have acted as a histamine antagonist, thereby reducing the spreading action of the hyaluronidase preparation. In order to investigate the above suggestion, a study, the results of which are reported in this paper, was made of the effect of salicylate on the skin-diffusing activity of hyaluronidase prepared from bull semen and of snake venom, together with its effect on the capillary-damaging action of these substances, and also of histamine alone and when mixed with hyaluronidase.

METHODS

Two experiments, similarly planned, were made: the first tested the spreading action and capillary-damaging effect of bull-testis hyaluronidase and snake venom before and after the administration of salicylate; the second tested the spreading effect of hyaluronidase and of hyaluronidase together with histamine, and the capillary-damaging action of histamine alone, before and after the administration of salicylate. In each experiment three albino rabbits weighing between 2 and 3·3 kg. were used. They were depilated several hours before the commencement of the experiment by means of a barium sulphide paste. Each rabbit received an intravenous injection of pontamine blue, 5% (w/v) in saline, 0·6 ml./kg. body weight, followed 15 min. later by the intradermal injection in 0·2 ml. quantities of the substances to be tested, the injections being confined to the left flank. Two injections of each substance were made in each rabbit, four substances being tested at a time, the arrangement of injections being completely randomized in eight approximately square areas marked out on the rabbits' flanks. Injections containing India ink in approximately 10% concentration were used to show up the spreading action, while injections devoid of India ink were used to demonstrate the capillary-damaging effect, manifested by the appearance of the blue dye. The average diameters of the areas of blue dye or India ink respectively were measured 30 min. after the intradermal injections. The rabbits were then given intravenous injections of sodium salicylate in 10% (w/v) solution, each receiving 0·1 g./kg. body weight. After 15 min., the intradermal injections were repeated on the right flank, the results being read after a further 30 min. as before.

In Exp. 1 the substances tested were bull-semen hyaluronidase, 10 units (Swyer & Emmens, 1947) in 200 ml. saline (0·1 ml. + 0·1 ml. saline or 0·1 ml. India ink 20% v/v in saline) and rattlesnake venom, 14 mg. in 40 ml. saline, this solution having the same viscosity-reducing activity as that of the bull hyaluronidase (0·1 ml. + 0·1 ml. saline or 0·1 ml. India ink). The arrangement of injections is illustrated in Table 1.

Table 1. *Illustration of arrangement of intradermal injections in Exp.* 1

(*a*) Mixtures

Substance	Symbol
Hyaluronidase + saline	1, 3
Hyaluronidase + India ink	2, 4
Snake venom + saline	5, 7
Snake venom + India ink	6, 8

(*b*) Positions

Head

Before salicylate		After salicylate	
7	6	8	1
3	2	7	3
5	8	5	6
1	4	2	4

In Exp. 2 the substances tested were bull-semen hyaluronidase as in Exp. 1 containing 20% (v/v) India ink (0·1 ml. + 0·1 ml. saline); histamine diphosphate 0·1 mg./ml. (0·1 ml. + 0·1 ml. saline); hyaluronidase + histamine (0·1 ml. of each); and saline (0·2 ml.).

Reading the results was complicated by the difficulty of separating, in some of the cases, the effect produced by the spread of India ink from that of staining with pontamine

blue caused by increased capillary permeability. It was decided, therefore, to measure in each case the maximum blue area regardless of whether due to India ink or pontamine blue. That this was a satisfactory criterion of the effects under consideration is shown by the analysis of variance (Tables 3 and 7).

RESULTS

Experiment 1. *Bull-testis hyaluronidase and snake venom, before and after salicylate*

The mean diameters of stained areas produced by these substances, and read as described above, are shown in Table 2. The analysis of variance for this experiment is set out in Table 3, from which it may be concluded that (1) there are significant differences in the reactions of different rabbits; (2) the responses to hyaluronidase and to snake venom show highly significant differences, and (3) the overall effect of salicylate is highly significant. In addition, the differential response with India ink and with pontamine blue to hyaluronidase is significantly different from that to snake venom, as is the differential response before and after salicylate to the two substances.

In order further to analyze these differential responses, the results were split so as to furnish analyses of variance for hyaluronidase and for snake venom separately; these are set out in Tables 4 and 5 respectively. From Table 4 it can be seen that the difference in response of pontamine blue and India ink to hyaluronidase is highly significant; in other words, as appears from Table 2, the hyaluronidase preparation had no significant effect on the pontamine blue reaction. The difference in response to hyaluronidase before and after the administration of salicylate is seen not to be significant. In the case of the response to snake venom, Table 5 shows that there was again a significant difference of response of pontamine blue and of India ink, though reference to Table 2 shows that snake venom, in contrast to the hyaluronidase preparation, produced a marked pontamine blue reaction. There was, moreover, a highly significant reduction of response to snake venom after the administration of salicylate.

Table 2. *Mean diameters in millimetres of stained skin areas in Exp.* 1

	Hyaluronidase + saline		Hyaluronidase + India ink		Snake venom + saline		Snake venom + India ink	
Rabbit	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate
1	0	5	12	7	24	20	20	17
	7	8	9	8	27	18	15	13
2	4	0	7	5	20	(14)	11	7
	0	0	9	5	22	12	10	9
3	0	0	10	6	24	22	15	10
	0	0	6	9	22	9	15	7
Totals	11	13	53	40	139	95	86	63

The value (14), missing from the data, was replaced by the method of least squares and one degree of freedom was dropped in the analysis of variance.

Table 3. *Analysis of variance for Exp.* 1

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
Rabbits (A)	2	188·5417	94·2709	11·98	<0·001*
Hyaluronidase *v.* snake venom (B)	1	1474·0834	1474·0834	187·31	<0·001*
Pontamine blue *v.* India ink (C)	1	5·3334	5·3334	—	—
Before salicylate *v.* after salicylate (D)	1	126·7501	126·7501	16·11	<0·001*
		Interactions			
A/B	2	18·2916	9·1458	1·16	>0·05
A/C	2	3·7916	1·8958	—	—
A/D	2	6·1249	3·0625	—	—
B/C	1	494·0832	494·0832	62·78	<0·001*
B/D	1	65·3332	65·3332	8·30	>0·001,* <0·01
A/B/C	2	14·0414	7·0207	—	—
A/B/D	2	8·0418	4·0209	—	—
A/C/D	2	5·3751	2·6876	—	—
B/C/D	1	27·0002	27·0002	3·43	>0·05
A/B/C/D	2	9·1252	4·5626	—	—
Error	23	181·0000	7·8696	—	—
Total	46	2627·6667	—	—	—

* Highly significant.

Table 4. *Analysis of variance for responses to hyaluronidase in Exp.* 1

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
Rabbits (A)	2	54·25	27·125	5·76	>0·01, <0·05
Pontamine blue *v.* India ink (C)	1	198·375	198·375	42·13	<0·001*
Before salicylate *v.* after salicylate (D)	1	5·0416	5·0416	1·07	>0·05
		Interactions			
A/C	2	14·25	7·125	1·51	>0·05
A/D	2	7·5834	3·7917	—	—
C/D	1	9·3751	9·3751	1·99	>0·05
A/C/D	2	9·2499	4·6250	—	—
Error	12	56·5	4·7083	—	—
Total	23	354·625	—	—	—

* Highly significant.

Table 5. *Analysis of variance for responses to snake venom in Exp.* 1

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
Rabbits (A)	2	152·5833	76·2917	6·74	>0·01, <0·05
Pontamine blue *v.* India ink (C)	1	301·0416	301·0416	26·60	<0·001*
Before salicylate *v.* after salicylate (D)	1	187·0416	187·0416	16·53	>0·001, <0·01*
		Interactions			
A/C	2	3·5834	1·7917	—	—
A/D	2	6·5834	3·2917	—	—
C/D	1	18·3751	18·3751	1·62	>0·05
A/C/D	2	5·2499	2·6250	—	—
Error	11	124·5000	11·3182	—	—
Total	22	798·9583	—	—	—

* Highly significant.

If one accepts the pontamine blue reaction as a reliable criterion of increased capillary permeability, the conclusion to be reached from the above results is (1) that a satisfactory comparison has been made of the action on capillaries and the skin-diffusing activity of hyaluronidase prepared from bull semen and of snake venom; (2) that salicylate had no effect on the skin-diffusing activity of hyaluronidase but did significantly reduce both the capillary-damaging and the skin-diffusing activities of snake venom.

Experiment 2. *Hyaluronidase alone and with histamine, before and after salicylate*

In view of the results obtained in Exp. 1, it was considered worth while to compare the spreading action of hyaluronidase with a hyaluronidase-histamine mixture, both before and after the administration of salicylate, since it seemed probable that part at least of the responses to snake venom could be attributable to the presence of histamine in the venom. By means of the technique previously described and used in Exp. 1, it was possible to test at one and the same time the effect of histamine, alone and in combination with hyaluronidase, on capillary permeability before and after the administration of salicylate.

The results of this experiment, given as mean diameters of stained areas, are shown in Table 6. The analysis of variance is set out in Table 7, from which it may be concluded (1) that differences between rabbits were significant, and (2) that the overall effects of hyaluronidase, histamine, and salicylate, were all highly significant. Furthermore, there was no significant interaction between histamine and hyaluronidase, which implies that their effects were strictly additive; the interaction between hyaluronidase and salicylate was significant but not great, while, in contrast, that between histamine and salicylate was highly significant. The somewhat artificial reduction in the sum of squares used as the error term by the inclusion of the six pairs of zeros contributed by saline alone accounts for the significance of the hyaluronidase and salicylate interaction. The other significant effects would, of course, remain significant with a much larger error term.

It is therefore to be concluded that the spreading action of hyaluronidase is increased in the predicted manner by the presence of histamine, and that, as in Exp. 1, salicylate scarcely influenced the spreading action of hyaluronidase, while, as can be seen by

Table 6. *Mean diameters in millimetres of stained skin areas in Exp.* 2

Rabbit	Hyaluronidase + India ink		Hyaluronidase + histamine + India ink		Histamine + saline		Saline	
	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate	Before salicylate	After salicylate
1	9	12	17	6	17	0	0	0
	7	9	30	11	19	0	0	0
2	9	9	25	12	19	0	0	0
	9	9	22	14	19	0	0	0
3	6	7	17	6	15	0	0	0
	8	10	20	7	14	0	0	0
Totals	48	56	131	56	103	0	0	0

Table 7. *Analysis of variance for Exp.* 2

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F*	*P*
Rabbits (A)	2	45·7917	22·8959	4·43	>0·01, <0·05
Hyaluronidase (B)	1	736·3334	736·3334	142·52	<0·001*
Histamine (C)	1	720·7501	720·7501	139·50	<0·001*
Salicylate (D)	1	602·0834	602·0834	116·53	<0·001*
		Interactions			
A/B	2	11·7916	5·8958	1·14	>0·05
A/C	2	22·8749	11·4375	2·21	>0·05
A/D	2	4·2916	2·1458	—	—
B/C	1	8·3332	8·3332	1·61	>0·05
B/D	1	26·9999	26·9999	5·23	>0·01, <0·05
C/D	1	720·7499	720·7499	139·50	<0·001*
A/B/C	2	3·0418	1·5209	—	—
A/B/D	2	2·6251	1·3126	—	—
A/C/D	2	7·8751	3·9376	—	—
B/C/D	1	8·3335	8·3335	1·61	>0·05
A/B/C/D	2	10·0415	5·0208	—	—
Error	24	124·0000	5·1667	—	—
Total	47	3055·9167	—	—	—

* Highly significant.

reference to Table 6, salicylate in the dosage used completely inhibited the capillary-damaging action of histamine diphosphate injected in 0·01 mg. amounts. The skin-diffusing action, moreover, of a hyaluronidase-histamine mixture, was reduced, by means of salicylate, to that of the hyaluronidase component alone.

DISCUSSION

Allusion has already been made to the discrepancy between the *in vivo* inhibitory effect of salicylate on the skin-diffusing activity of hyaluronidase described by Guerra (1946*a*, *b*) and the failure of *in vitro* inhibition of viscosity-reducing activity reported by Swyer (1948), as well as to the tentative explanation offered. The evidence presented in this paper regarding the potentiation of hyaluronidase skin-diffusing activity by histamine and its inhibition by salicylate lends substantial support to such an explanation. At the same time, the effect of salicylate in preventing increase of capillary permeability by histamine is in itself a finding of considerable interest.

If, as seems to be clear, the anti-rheumatic action of salicylates is not due, as Guerra proposed, to their alleged inhibitory effect on hyaluronidase, the question naturally arises as to whether it can be ascribed to their anti-histamine properties. This problem is briefly discussed by Swyer (1948), but the conclusion is reached that the evidence available in the literature on the effects of salicylate on immunological reactions, though fairly extensive, is scarcely sufficient to afford a solution. At the same time, however, such an interesting possibility cannot be excluded.

SUMMARY

1. Using factorial tests on rabbits, comparisons have been made of the skin-diffusing activities of hyaluronidase prepared from bull semen and rattlesnake venom, and their effects in increasing capillary permeability as evidenced by the pontamine blue reaction, both before and after the administration of

sodium salicylate. In the same way, the effect of salicylate on the skin-diffusing and capillary-damaging actions of hyaluronidase, histamine and a hyaluronidase-histamine mixture have been studied.

2. The hyaluronidase preparation had no significant effect on capillary permeability, and its spreading action was not affected by salicylate. Snake venom, on the other hand, had a marked capillary-damaging effect which, along with its spreading action, was significantly reduced by salicylate. Histamine increased the spreading action of hyaluronidase in a strictly additive fashion, and its capillary-damaging action was inhibited by salicylate to a highly significant extent; salicylate reduced the spreading action of a hyaluronidase-histamine mixture to that of the hyaluronidase component alone.

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REFERENCES

Guerra, F. (1946*a*). *Science*, **103**, 686.
Guerra, F. (1946*b*). *J. Pharmacol.* **87**, 193.
Swyer, G. I. M. (1948). *Biochem. J.* **42**, 32.
Swyer, G. I. M. & Emmens, C.W. (1947). *Biochem. J.* **41**, 29.

Failure of *in vitro* Inhibition of Hyaluronidase by Salicylates

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Guerra (1946 *a*, *b*) claimed that sodium salicylate in doses of 0·07 and 0·10 g./kg. caused 57 and 66% reduction of the skin diffusion effect of hyaluronidase in rabbits. Moreover, he reported that in humans who had active rheumatic fever, or had suffered it, intradermal injections of hyaluronidase with Evans blue gave 'unique reactions with enormous diffusion of the dye and local oedema that sometimes occupies the arm injected with hyaluronidase'. Salicylates in these cases inhibited the enzyme and reduced its spreading effect in connective tissues. On the basis of these findings he believed that hyaluronidase played an important part in the pathogenesis of rheumatic fever and that the anti-rheumatic action of salicylates could be explained by their inhibition of the enzyme.

Since considerable and increasing attention is being paid to these reports, it seems proper to record the results of some experiments on the *in vitro* effect of salicylates on hyaluronidase action, in which, using a viscosimetric technique, the accuracy of which is far greater than that employing skin diffusion, no direct inhibitory effect was observed except with relatively enormous salicylate concentrations. At the same time, the action of salicylates will be compared with that of heparin, a true hyaluronidase inhibitor (McClean, 1942; Rogers, 1946).

METHODS

The effects of sodium salicylate, acetylsalicylic acid and heparin on the activity of hyaluronidase in decreasing the viscosity of buffered solutions of potassium hyaluronate were studied by a modification of the hyaluronidase assay technique described by Swyer & Emmens (1947). The enzyme preparation was the standard freeze-dried bull-semen filtrate described by those authors. Activity of the enzyme is expressed by the flow-time index, which has been shown to be proportional over a fairly wide range to the logarithm of the concentration of the enzyme. In all cases, the reaction mixture consisted of 0·2% potassium hyaluronate, 1 ml.; McIlvaine's citric acid phosphate buffer, M/60, pH 7·0, containing NaCl (3·5 g./l.), 0·5 ml.; appropriate dilution of the substance to be tested, 1 ml.; enzyme solution, 0·5 ml. The reaction took place, and the flow-time through the viscosimeter tube was read (20 min. after adding enzyme to substrate) at 34° (see Swyer & Emmens (1947) for details).

RESULTS

In the first test, serial dilutions of 1% (w/v) sodium salicylate solution were tested against enzyme solutions containing 0·5 unit (Swyer & Emmens, 1947) and 0·125 unit/ml. The results, shown in Table 1, indicate that over this range of concentration of salicylate no inhibition occurs, while with 0·33% salicylate in the reaction mixture there appeared to be facilitation.

Table 1. *Effect of sodium salicylate on hyaluronidase action*

Na salicylate in reaction mixture (%)	pH	Flow-time index (Hyaluronidase, unit/ml.) (0·125)	(0·25)	(0·5)	Notes
0	—	605	—	872	Control
0·003	—	677	—	834	No inhibition
0·033	—	624	—	860	
0·066	—	605	—	872	
0·16	—	644	—	872	
0·33	—	733	—	904	Facilitation
0	7·0	560	765	—	Control
0·33	7·0	566	890	—	Facilitation
0·833	7·0	653	835	—	
1·66	6·6	276	515	—	Inhibition
2·5	6·4	133	292	—	
3·33	6·2	150	318	—	

The tests were repeated with dilutions of 10% sodium salicylate, using enzyme solutions containing 0·25 and 0·125 unit/ml. The results (Table 1) confirm the facilitation effect over the range of salicylate concentrations 0·33–0·83% in the reaction mixture. When the salicylate concentration rose to 1·66% and higher, increasing inhibition was observed, but it was also found that the pH of the reaction mixture fell progressively with increasing salicylate concentration (see below).

Acetylsalicylic acid was first tested in serial dilutions of a 1% (w/v) solution against an enzyme solution containing 0·5 unit/ml. Complete inhibition occurred when the concentration in the reaction mixture reached 0·33%. Further tests with concentrations ranging between 0·066 and 0·33% against enzyme solutions containing 0·125 and 0·25 unit/ml. revealed increasing inhibition over this range (Table 2). The pH of the reaction mixtures in one of these latter tests was estimated with indicators using the British Drug Houses capillator outfit, and equivalent mixtures were made, using acetic acid in place of acetylsalicylic acid, so as to obtain as near as possible the same pH. The degree of enzyme activity in these mixtures resembled quite closely that found in the former ones, thus indicating that the observed inhibitory effect could be explained simply on the basis of hydrogen ion concentration (Table 2). As can be seen from Table 1, the fall of pH with strong sodium salicylate mixtures was considerably less marked than with acetylsalicylic acid; the greatly increased molarity of the mixture is probably to be held partly responsible for the inhibitory effect with strong sodium salicylate mixtures.

The effect of heparin on hyaluronidase activity was investigated by using serial dilutions of a solution of heparin containing 10 mg. (101 Toronto units/mg.) in 10 ml. water in the same way as previously described, the hyaluronidase solution

Table 2. *Effect of acetylsalicylic acid and acetic acid on hyaluronidase action*

(*a*) Addendum: acetylsalicylic acid

Acetylsalicylic acid in reaction mixture (%)	pH	Flow-time index (Hyaluronidase, 0·5 unit/ml.)	(Hyaluronidase, 0·125 unit/ml.)	(Hyaluronidase, 0·25 unit/ml.)
0	—	872		
0·003	—	898		
0·033	—	840		
0·066	—	848		
0·16	—	645		
0·33	—	0*		
0	7·0		560	775
0·066	6·6		433	545
0·133	5·4		388	297
0·2	5·0		174	166
0·266	4·2		66	157
0·33	3·8		0*	111

(*b*) Addendum: acetic acid

	(Hyaluronidase, 0·25 unit/ml.)
7·0	—
6·6	505
5·4	350
5·0	155
4·2	123
3·8	0*

* Complete inhibition.

containing 0·25 unit/ml. From Table 3 it can be seen that inhibition is complete when the reaction mixture contains 6·6 units of heparin/ml. (i.e. 0·0066%), while below this concentration the degree of inhibition decreases until, with 0·163 unit/ml. in the reaction mixture, it disappears. In no case was the pH of the reaction mixture altered from its initial value of 7·0.

Table 3. *Effect of heparin on hyaluronidase action*

Heparin: (units/ml. in reaction mixture)	Flow-time index (Hyaluronidase, 0·25 unit/ml.)	Notes
0	775	Control
0·033	790	No inhibition
0·163	800	
0·33	405	Increasing inhibition
1·63	375	
3·3	152	
6·6	0	Complete inhibition

Table 4. *Combined effect of variation of heparin and hyaluronidase concentrations—factorial scheme showing viscosity index for each of sixteen combinations of hyaluronidase and heparin*

Hyaluronidase (unit/ml.)	Heparin (units/ml.) 40	20	10	5
0·125	0	0	0	96
0·25	0	0	72	134
0·5	0	85	102	420
1·0	195	273	495	745

Flow-time indices

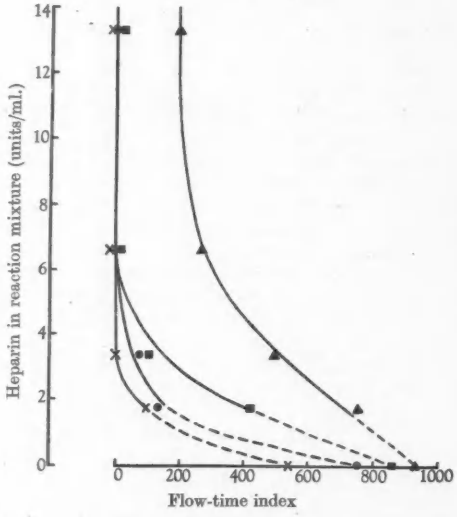

Fig. 1. Effect of concentration of heparin on the viscosity reducing activity of hyaluronidase solutions of different strengths: ×—× 0·125 unit/ml.; ●—● 0·25 unit/ml.; ■—■ 0·5 unit/ml.; ▲—▲ 1·0 unit/ml.

The effect of combined variation of heparin and of enzyme concentration was studied by means of a factorial test involving four concentrations of heparin and four of enzyme, the sixteen combinations being tested in random order (Table 4). The results are illustrated by Fig. 1, in which the flow-time index is plotted against concentration of heparin for the four concentrations of enzyme; it is clear that a relationship exists between quantity of heparin and amount of enzyme inhibited, whereas in the case of salicylates, the evidence suggested that no such relationship held.

DISCUSSION

Comparison of the effects of salicylates with those of heparin on the viscosity-reducing activity of hyaluronidase shows quite clearly the difference between a true hyaluronidase inhibitor, heparin, which, in a concentration of 0·0066 % in the reaction mixture, completely inhibits the activity of an enzyme solution containing 0·25 unit/ml. without altering the pH, and the salicylates, whose inhibitory action, manifested only in very high concentrations (more than 3 % for sodium salicylate and 0·33 % for acetylsalicylic acid), appears to be directly due to their effect on the pH of the reaction mixture, or, in the case of sodium salicylate, to the combined effect of lowered pH and increased salt concentration. Since, however, a close correlation has been shown to exist between the skin-diffusing and viscosity-reducing activities of hyaluronidases (McClean, 1943; Humphrey, 1943), it is surprising that sodium salicylate should possess the inhibitory effect on skin diffusion as described by Guerra. The recent work of Hechter (1946, 1947) may help to provide an explanation for this discrepancy. This investigator has shown very clearly that the skin-diffusing activity of hyaluronidase depends not only on the concentration of the enzyme, but also on the volume of solution injected, or on the interstitial fluid pressure; if the latter be maintained as a result of increased capillary permeability (such as is produced by histamine and inflammation-inducing agents) the extent of spread is considerably greater than when there is no accompanying capillary change. It is suggested, therefore, that the hyaluronidase preparation used by Guerra contained histamine, or some substance with a similar action, and that sodium salicylate, by inhibiting this substance, reduced the apparent spreading effect of hyaluronidase. The validity of such an explanation is rendered more probable by the observations (Swyer, 1948) that histamine enhances the spreading action of hyaluronidase, as could be predicted from the findings of Hechter (1946, 1947), and that sodium salicylate in the dose employed by Guerra (0·1 g./kg. intravenously in rabbits) inhibits the ability of histamine to increase capillary permeability and thereby reduces the spreading action of a hyaluronidase-histamine mixture.

In view of the evidence which has been presented that hyaluronidase is not inhibited by salicylates in therapeutic concentrations, it would seem that Guerra's explanation of the anti-rheumatic action of these drugs in terms of hyaluronidase inhibition must be abandoned. The question naturally arises as to whether or no their apparent anti-histamine action can be invoked in this connexion, as well as whether the effect of hyaluronidase on the skin of rheumatic fever patients described by Guerra and its diminution after administration of salicylate can be explained by the same mechanism. A voluminous literature exists regarding the effects of salicylates on immunological reactions, but the evidence, though some is suggestive, seems to be scarcely sufficient to provide a complete answer to these questions. The response to hyaluronidase described by Guerra in rheumatic fever, however, appears to be of the nature of a hypersensitivity reaction, such as was described by Jones & Mote (1934) as occurring in rheumatic fever patients following repeated injections of very small amounts of rabbit serum. It is true that only a single injection of hyaluronidase appeared to have been given (in Guerra's paper no details are provided, nor is any mention made of control tests in normal subjects), but since streptococci of the type believed to be responsible for predisposing to rheumatic fever are hyaluronidase producers, sensitivity to hyaluronidase might well be of general occurrence in rheumatic fever. If such be the case, inhibition of histamine, which is believed to play a part in such hypersensitivity reactions, would account for the reduced response to intradermal injections of hyaluronidase after treatment with salicylate.

SUMMARY

1. Investigation of the effects of sodium salicylate and acetylsalicylic acid on the viscosity-reducing activity of hyaluronidase has shown that they are inhibitory only in relatively enormous concentrations (more than 3 % for sodium salicylate and 0·33 % for acetylsalicylic acid).

2. Such inhibition is apparently due to lowering of pH and, in the case of sodium salicylate, to increased salt concentration.

3. Heparin, on the other hand, is completely inhibitory in a concentration of 0·0066 %.

4. The significance of these findings in relation to the claims made by Guerra (1946*a*) of the association of hyaluronidase with rheumatic fever, and the anti-rheumatic action of salicylates is discussed.

My sincere thanks are due to Drs C. W. Emmens and J. H. Humphrey for advice generously given.

REFERENCES

Guerra, F. (1946*a*). *Science*, **103**, 686.
Guerra, F. (1946*b*). *J. Pharmacol.* **87**, 193.
Hechter, O. (1946). *Science*, **104**, 409.
Hechter, O. (1947). *J. exp. Med.* **85**, 77.
Humphrey, J. H. (1943). *Biochem. J.* **37**, 177.
Jones, T. D. & Mote, J. R. (1934). *New Engl. J. Med.* **210**, 120.
McClean, D. (1942). *J. Path. Bact.* **54**, 284.
McClean, D. (1943). *Biochem. J.* **37**, 169.
Rogers, H. J. (1946). *Biochem. J.* **40**, 583.
Swyer, G. I. M. (1948). *Biochem. J.* **42**, 18.
Swyer, G. I. M. & Emmens, C. W. (1947). *Biochem. J.* **41**, 29.

The Influence *in vitro* of Deoxycorticosterone on Glycogen Formation in Muscle

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The main action of the adrenal cortical hormone is generally believed to be the regulation of glycogen metabolism. In a series of experiments from this Institute the phosphorylation of glycogen was investigated in normal and adrenalectomized animals. This reaction, by which glucose-1-phosphate is formed from glycogen, is the first phase in the breakdown of glycogen. It was shown that the phosphorylation of glycogen *in vitro* was greatly reduced in tissue from adrenalectomized animals (Verzár & Montigel, 1942; Doetsch, 1945).

In order to study directly the synthesis of glycogen, we chose the technique of Gemmill (1940, 1941) and Gemmill & Hamman (1941) by which the synthesis of glycogen from glucose can be observed in the surviving diaphragm of the rat. The rat's diaphragm is suitable for this experiment because, owing to its thin structure, it can be kept alive in Ringer solution for a few hours if saturated with oxygen. If glucose is added to this solution, the diaphragm synthesizes glycogen. Gemmill (Gemmill, 1940, 1941; Gemmill & Hamman, 1941) and also

Hechter, Levine & Soskin (1941) have shown that this glycogen synthesis can be considerably increased by the addition of insulin. The diaphragm of adrenalectomized rats, too, synthesized glycogen from glucose, and insulin gave the same increase as in normal rats (Koepf, Horn, Gemmill & Thorn, 1941). It thus appeared that the main disturbance in glycogen metabolism in adrenalectomized animals is not in the synthesis of glycogen.

We therefore investigated the effect of the addition of deoxycorticosterone (DOC) on the glycogen formation in the diaphragm of normal and adrenalectomized rats. We found that DOC inhibits completely the formation of glycogen and also the effect of insulin on this formation.

METHODS

The method of Gemmill (1940, 1941) was used with slight modification, but concentrations of insulin and glucose were kept within physiological limits. Rats of 100–150 g. body weight of both sexes and of our own breed were killed by a blow on the head and decapitation and were bled as much as possible. The diaphragm was rapidly dissected out and cut into three parts of almost equal weight (from 100 to 150 mg.). The posterior thicker part of the diaphragm was immediately weighed on a torsion balance, dropped into 20% (w/v) NaOH (1 ml.) and hydrolyzed on a boiling water bath for the estimation of the initial glycogen content. The two remaining pieces of diaphragm were also weighed immediately, washed in phosphate buffer Ringer solution and dropped into 50 ml. Erlenmeyer flasks containing 10 ml. of the phosphate buffer Ringer solution, which was prepared according to Hastings, Muus & Bessey (1939) and contained 8 g. NaCl, 0·2 g. KCl, 0·2 g. $CaCl_2$, 0·1 g. $MgCl_2$, 0·8 g. $Na_2HPO_4.2H_2O$ and 0·15 g. KH_2PO_4/l. (pH *c.* 7·2–7·4 as measured by Bayer pH papers nos. 7 and 8). The following substances were then added to the Ringer solution: 100 mg. glucose/100 ml., 1 unit insulin/100 ml., 0·01–10 mg. DOC/100 g. and 0·25–0·50% ethanol. A solution of insulin (Eli Lilly) with an activity of 40 units/ml. was diluted with Ringer solution to a concentration of 1 unit/ml. Usually 0·1 ml. of this solution was dropped into the flasks containing 10 ml. of the phosphate buffer Ringer solution. The final insulin concentration was therefore 1 unit/100 ml. DOC (100 mg.) was dissolved in 5 ml. ethanol and 5 ml. water were added; 0·1 ml. of this solution was added to the flasks if a concentration of 10 mg./100 ml. was required. According to Miescher & Meystre (1943) DOC has a solubility of 0·012% in water and so all the DOC in our experiments was dissolved by the Ringer solution. Solutions of 0·1 and 0·01% DOC were used if lower concentrations were needed. If diaphragm is permeable to insulin and DOC, the hormone concentrations in the muscle and in the Ringer solution should be identical. One of the lateral pieces of diaphragm was always kept as a control without the addition of the hormone. The flasks containing the pieces of diaphragm were at once filled with oxygen, closed with rubber stoppers and shaken in a water bath at 37°. From time of death to the beginning of shaking only 4–5 min. elapsed. It was therefore not necessary to prepare the diaphragms at 0°. In the majority of experiments the shaking in the water bath was continued for 1·5 hr., and the pieces of diaphragm were then dropped into 1 ml. of 20% (w/v) NaOH and hydrolyzed on a boiling water bath until the solution was clear (10–15 min.). The glycogen was then precipitated from the hot solution with 2 ml. of ethanol and the precipitation completed by standing in ice water for 0·5 hr. The glycogen was centrifuged off, the supernatant poured away, the glycogen stirred up with 2 ml. of ethanol, again centrifuged at 3000 r.p.m. for 3–5 min. and dried on a boiling water-bath. According to the amount of precipitate 1–2 ml. 2N-H_2SO_4 were added and the glycogen hydrolyzed on a boiling water bath for 1·5 hr. The hydrolysate was washed into a 50 ml. measuring flask and made alkaline with 2·3 ml. of 2N-Na_2CO_3. Depending on the amount of glycogen 2–10 ml. were taken from the flask and the sugar content estimated by the Hagedorn-Jensen method, according to Hallmann (1939). The glycogen contents are given as mg. glycogen/100 g. wet muscle.

RESULTS

The results are reported in Tables 1 and 2. Only the mean values of results obtained with several animals are given. The glycogen contents are given at the beginning of the experiment (initial glycogen content) and after 1·5 hr. with additions of the different substances as glucose, insulin, DOC and ethanol. The tables show the differences between the glycogen contents before and after incubation with a given addition. More important, however, are the differences between the results with different additions, i.e. glucose and glucose + insulin, or no glucose and glucose + insulin, or those with DOC, which give the true effect of the addition. From Tables 1 and 2 these values can easily be calculated. Since all values are arithmetic mean values the standard error for the differences in each series has been calculated. One example of a series with 10 single experiments is given in Table 2. Here the differences between the results with different additions are also shown.

1. *Action of glucose*

The experiments on 25 animals (Table 1, series 1) show that the glycogen content of the diaphragm in phosphate buffer Ringer solution without glucose decreased in 1·5 hr. by a mean value of 57 mg./100 g. Addition of glucose (100 mg./100 ml.) (without insulin) produced glycogen in normal rats (series 2) and also in rats which had been starved for 2 days (series 6). In the former series the glycogen content increased by 46 mg./100 g. compared with the initial value and in the latter by 126 mg./100 g. If these values are compared with those of series 1 (without glucose), where the glycogen content decreased by 57 mg./100 g., the total glycogen production can be calculated to be 103 and 183 mg./100 g. respectively. In single experiments differences of 50 mg./100 g. are not regarded as real, but in a series of several experiments the mean error becomes much smaller, as is shown in the tables.

Table 1. *Changes in the glycogen contents of diaphragms of normal, starved and adrenalectomized rats on incubation with and without the addition of glucose, insulin and deoxycorticosterone*

Series no.	No. of rats	Mean initial glycogen content (mg./100 g. of muscle)	Additions to suspending fluid: Glucose (mg./100 ml.)	Insulin (units/100 ml.)	DOC (mg./100 ml.)	Ethanol (%)	Mean glycogen content after 1·5 hr. (mg./100 g. of muscle)	Mean differences (mg./100 g. of muscle)	S.E. of mean differences
1	25	239	—	—	—	—	182	− 57	12
2	60	280	100	—	—	—	326	+ 46	13
3	13	296	100	0·1	—	—	397	+101	20
4	14	265	100	0·5	—	—	494	+229	17
5	102	233	100	1	—	—	363	+130	8
6	13*	100	100	—	—	—	226	+126	8
7	13*	100	100	0·5	—	—	422	+322	19
8	8	170	—	0·5	—	—	136	− 34	23
9	11	165	—	1	—	—	142	− 23	9
10	8	129	100	100	—	—	279	+150	17
11	8	270	100	—	10	0·50	185	− 85	22
12	14	305	100	—	5	0·25	298	− 7	14
13	12	301	100	—	1	0·50	297	− 4	16
14	11	261	—	—	5	0·25	203	− 58	13
15	14	326	100	—	—	0·50	377	+ 51	20
16	10	175	100	1	—	0·50	321	+146	24
17	12	187	100	—	10	—	155	− 32	14
18	12	277	100	1	10	—	245	− 32	24
19	13	157	100	1	10	0·50	88	− 69	17
20	30	246	100	1	5	0·25	244	− 2	17
21	20	321	100	1	1	0·50	470	+149	20
22	6	203	100	1	0·5	0·25	318	+115	31
23	15	183	100	1	0·1	0·50	291	+108	28
24	6	131	100	1	0·05	0·25	290	+159	25
25	12	265	100	1	0·01	0·25	399	+134	25
26*a*	7	297	100	1	10	0·50	166	−131	39
26*b*	7	297	100	100	10	0·50	191	−106	32
27*a*	23†	225	100	—	—	—	288	+ 63	18
27*b*	23†	225	100	0·5	—	—	407	+182	15
28*a*	16†	164	100	1	—	—	282	+118	20
28*b*	16†	164	100	1	10	0·50	106	− 58	12

* After a starvation period of 2 days.

† Adrenalectomized animals.

2. *Action of insulin on glycogen production*

In Table 1, series 3, 4 and 5 show the effect on glycogen formation of 0·1, 0·5 and 1 unit of insulin/100 ml. respectively. To 10 ml. phosphate buffer Ringer solution in which was suspended one third of a diaphragm 0·1 unit of insulin was added (series 5). This corresponds to an insulin content of 1 unit/100 ml. of Ringer solution, or 100 g. of muscle. Insulin already at a dose of 0·1 unit/100 ml. increases glycogen production by about 50 mg./100 g. (series 3) as compared with glucose alone (series 2). With 0·5 unit the insulin effect is almost maximal giving an increase of 183 mg./100 g. as compared with glucose alone (series 4). In series 5, 102 experiments with insulin (1 unit/100 ml.) are collected. The mean value for the glycogen production is 130 mg./100 g. The values of this series are also plotted against the number of animals in Fig. 1. In this figure the total number of experiments in which a given increase (± 20 mg./100 g.) in glycogen content

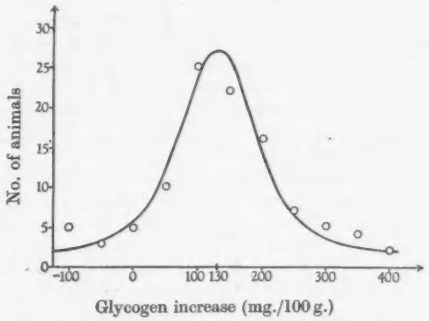

Fig. 1. Glycogen production from glucose (100 mg./100 ml.) by diaphragms of normal rats with insulin (1 unit/100 ml.) in 102 experiments. The ordinate value of each point indicates the number of diaphragms which produced the amount of glycogen (±20 mg./100 g. muscle) given by the abscissa. The solid line is the normal probability equation.

was obtained is plotted, as the ordinate, against the magnitude of the given increase (abscissa).

Nearly 80 % of all experiments fall inside the values of +50 to +300 mg./100 g. There are, however, even in this series a certain number of negative results which are included in the mean value decreasing this to 130 mg./100 g. This seems to be lower than the mean value with 0·5 unit insulin/100 ml. in a series with only 14 experiments, but the difference is not significant.

In the experiments of Gemmill and co-workers (Gemmill, 1940, 1941; Gemmill & Hamman, 1941; and Koepf *et al.* 1941), 100–200 units insulin/100 ml. were used, but these authors observed about the same maximal increase of glycogen production as we found with much smaller quantities of insulin. We have therefore compared the action of different quantities of insulin on two parts of the same diaphragm. (The third part of the diaphragm was always used to obtain the initial glycogen value.)

In a series of 4 experiments we found glycogen productions, with 0·5 unit insulin/100 ml., of 65 mg./100 g. and, with 1 unit/100 ml. of 70 mg./100 g. In another series of 8 experiments we found glycogen productions, with 1 unit/100 ml. of 136 mg./100 g. and, with 100 units/100 ml. of 150 mg./100 g. (series 10).

Thus, increasing the concentration of insulin above 0·5 unit/100 ml. has very little effect, and on the same diaphragm 0·5 unit acts like 1, or 1 like 100 units/100 ml. It must be remembered however that the activity of different doses of insulin cannot be compared on different diaphragms. The largest values for glycogen content, above 400 mg./100 g., were found with 0·5 unit/100 ml. in series 4 and 7, and these are not less than with 100 units/100 ml. in series 10.

We have also measured the time in which insulin has this effect. Insulin (1 unit/100 ml.) was added to both parts of the diaphragm and the incubation was stopped at different times. Glycogen production is especially active in the first 0·5 hr. being about 100 mg./100 g. After that, only about 10–20 mg./100 g. glycogen is formed in each 0·5 hr.

Control experiments were also done to see whether insulin has any action if it is added without glucose. Series 8 and 9 show that no glycogen was formed and the initial glycogen was broken down as in series 1 where neither glucose nor insulin had been added. Thus insulin cannot inhibit glycogenolysis.

Mention must be made of series 6 and 7 in which animals starved for 2 days were used. Such control experiments were necessary, since results obtained with adrenalectomized rats might be ascribed to the fact that they were starving. The glycogen content of the muscles in these animals was very low, about 100 mg./100 g. The glycogen content after addition of glucose alone was 226 mg./100 g., with glucose and insulin (0·5 unit/100 ml.) 422 mg./100 g. The insulin effect is 196 mg./100 g. (difference: 322 − 126 = 196) and is equal to that of animals on a normal diet (see series 2 and 4, difference: 229 − 46 = 183).

3. *Action of deoxycorticosterone*

We tested next the action of DOC with and without glucose and with and without insulin. The results are shown in series 11–18 (Table 1). In series 11, 12 and 13 the action of 10, 5 and 1 mg. DOC/100 ml. of solution (or g. of muscle) in the presence of glucose is shown. In all experiments no glycogen was produced from glucose. In series 11 there was even a breakdown of 85 mg./100 g. as compared with the initial glycogen content. If we compare series 2, in which a glycogen production of 46 mg./100 g. from glucose was demonstrated, the real decrease in glycogen in series 11 is 131 mg./100 g. In series 12 and 13 with 5 and 1 mg. DOC/100 ml. only the glucose effect was abolished by DOC. In series 14 no glucose but 5 mg.DOC/100 ml. were added and a decrease of glycogen of 58 mg./100 g. resulted, which is very similar to the decrease without any addition (series 1).

After addition of DOC the Ringer solution contains 0·25–0·50 % ethanol, as pointed out in the Methods section. Series 15 and 16 show that ethanol without DOC has no action whatever on the glycogen formation with glucose and with glucose + insulin. The results are the same as in series 2 and 5.

Experiments with ethanol (1 %), glucose (100 mg./100 ml.) and insulin (1 unit/100 ml.) showed a diminished glycogen production (by about 30 mg./100 g.) in comparison with glucose and insulin alone; 0·5 % ethanol, the concentration used in our previous experiments with DOC, had no action. Nevertheless we now tested whether DOC has an inhibitory action on glycogen production in aqueous solution, i.e. without ethanol. It is possible to dissolve about 0·01 % DOC in water (Miescher & Meystre, 1943). We took 100 mg. of finely pulverized crystalline DOC and shook it for 3 hr. in 500 ml. of phosphate buffer Ringer solution, which thus became saturated with DOC. After addition of 100 mg. glucose/100 ml. we dropped the diaphragms into 10 ml. of this solution and performed the experiment in the usual manner. The results are shown in series 17. With glucose and without insulin, DOC caused a glycogen breakdown of 32 mg./100 g., or 78 mg./100 g. when compared with series 2. The inhibition of glycogen production and the increase of glycogen breakdown by DOC is of the same order as that caused by DOC with 0·5 % ethanol (series 11 and 19). Thus ethanol in the concentrations used by us does not influence the inhibitory action of DOC on glycogen production.

All these experiments show that DOC does not increase glycogen formation in muscle, but rather inhibits glycogen production from glucose.

4. *Combined action of deoxycorticosterone and insulin*

Since the preliminary experiments of sections 1–3 have shown the smallest effective doses of insulin on glycogen formation, and of DOC on glycogen breakdown, we can describe our main experiment, i.e. the action of DOC in the presence of insulin.

In Table 1 these results are shown as mean values in series 19–26*b*. From series 19 it is obvious that, in the presence of 100 mg. glucose/100 ml. and 1 unit insulin/100 ml. with 10 mg. DOC/100 ml., no glycogen is formed but as much is broken down as if no glucose and no insulin were present. In series 20 with 5 mg. DOC/100 ml., less action is seen; there is no production of glycogen but also no decrease. In series 21 with 1 mg. DOC/100 ml., there is an increase of glycogen content, i.e. the action of insulin is not antagonized. Smaller DOC concentrations of 0·5–0·01 mg./100 ml. in the series 22–25 are equally without antagonistic action. In these the results are the same as with insulin alone; about 100–150 mg. glycogen/100 g. are formed. In all the series a corresponding amount of ethanol was added to the controls without DOC. In all cases ethanol in concentrations of 0·25 and 0·50 % was quite without influence on glycogen synthesis and breakdown.

In series 18 an aqueous solution of DOC without ethanol was used, parallel to series 17 mentioned above. Glycogen was broken down at the same rate as in series 19.

Series 19–21 contain the main results of this paper. We give therefore 10 consecutive single experiments from series 19 in Table 2, as an example of each series with its controls.

From these single experiments the same follows as from the mean values of Table 1. In the last column the total breakdown of glycogen is calculated by comparing the two glycogen contents after 1·5 hr., those with glucose and insulin alone and those with additional DOC. With 10 mg. DOC/100 ml. the effect on glycogenolysis was 174 mg./100 g. as mean value of these 10 experiments. In Table 1 comparing series 5 and 19 we calculate a DOC effect on glycogen breakdown of 199 mg./100 g. The single differences in each of the columns 2–1 and 3–1 vary considerably. Nevertheless except with the second experiment all differences in column 3–2 are very similar.

The effect of DOC on glycogen breakdown is not a direct antagonistic action against added insulin, as is shown by a comparison with series 11 where glycogen is broken down with DOC without insulin, as well as with DOC and insulin in series 19.

The series 26*a* and 26*b* prove the same. There is no correlation between the doses of insulin and DOC, because 1 unit insulin/100 ml. as well as 100 units/100 ml. is inhibited by 10 mg. DOC/100 ml.

5. *Action of insulin and DOC on the diaphragm of adrenalectomized adynamic rats*

The action of DOC was next studied with muscle from adrenalectomized animals.

Male rats, of 100–150 g. body weight, of the Institute's own breed, were adrenalectomized. They recovered from the operation in 3 days and increased in weight. The animals were killed between the 5th and 10th day if a decrease in weight and adynamia had appeared.

Series 27*a*–28*b* (Table 1) summarize the experiments by showing mean values. Series 27*b* and 28*a* confirm what was already known from Gemmill's experiments (Koepf *et al.* 1941), that the diaphragm of adrenalectomized rats *in vitro* produces glycogen in the same way as that of normal animals. If glucose only was present (series 27*a*), 63 mg. glycogen/100 g. were formed. With 0·5 unit insulin/100 ml. (series 27*b*) 182 mg./100 g. and with 1 unit/100 ml. (series 28*a*) 118 mg. glycogen/100 g. were produced.

Table 2. *Typical experiments demonstrating the action of deoxycorticosterone* (10 *mg./100 ml.*) *on glycogen formation by diaphragms from glucose and insulin*

Initial glycogen content (mg./100 g.)	Glycogen content after 1·5 hr. (mg./100 g.)		Differences in glycogen content (mg./100 g.)		
	With glucose and insulin	With glucose, insulin and DOC			
1	2	3	2–1	3–1	3–2
160	240	40	+ 80	−120	−200
140	40	40	−100	−100	0
80	230	40	+150	− 40	−190
370	370	160	0	−210	−210
180	360	110	+180	− 70	−250
40	250	70	+210	+ 30	−180
160	290	80	+130	− 80	−210
180	360	110	+180	− 70	−250
90	200	80	+110	− 10	−120
160	290	160	+130	0	−130
Means: **156**	**263**	**89**	**+107**	**− 67**	**−174**

If, however, in series 28*b* to 1 unit insulin/100 ml. 10 mg. DOC/100 g. were added, the glycogen content decreased by 58 mg./100 g. Thus the results were exactly the same as in normal animals in the series 2, 4, 5, 18 and 19.

6. *Action of insulin and DOC on glycogen formation from glucose-1-phosphate*

The experiments on glycogen formation by normal diaphragms were repeated using, instead of glucose, glucose-1-phosphate (Cori ester) as substrate. This substance, according to present knowledge, is the first product of glycogen breakdown, and possibly also the last intermediate in glycogen synthesis. Cori, Schmidt & Cori (1939) and others have shown that *in vitro* an enzyme fraction of muscle extracts, the so-called phosphorylase, can synthesize glycogen from glucose-1-phosphate.

The glucose-1-phosphate which was used for these experiments was prepared according to Cori, Colowick & Cori (1937). For these experiments the concentration used was equivalent to 100 mg. glucose/100 ml. (150 mg. glucose-1-phosphate/100 ml.). This was verified after hydrolysis by estimation of glucose by the Hagedorn-Jensen method.

Table 3. *Glycogen content of diaphragm from normal animals after incubation with glucose-1-phosphate*

Series no.	No. of rats	Mean initial glycogen content (mg./100 g. of muscle)	Additions to suspending fluid: Glucose-1-phosphate (mg./100 ml.)	Insulin (units/100 ml.)	DOC (mg./100 ml.)	Ethanol (%)	Mean glycogen content after 1·5 hr. (mg./100 g. of muscle)	Mean differences (mg./100 g. of muscle)	S.E. of mean differences
29	16	329	150	—	—	—	282	− 47	25
30	8	231	150	1	—	—	225	− 6	27
31	8	425	150	—	10	0·50	285	−140	53

The results are shown in Table 3. In series 29 (with glucose-1-phosphate alone) the glycogen decreased in the same way as in series 1 (Table 1) without glucose. Even with 1 unit insulin/100 ml. added there was no glycogen synthesis. DOC (5 mg./100 ml.) increased the glycogenolysis, as series 31 shows.

Thus glucose-1-phosphate cannot act as substrate for glycogen formation with intact muscle. Since Verzár & Wenner (1948) have found that minced muscle can synthesize glycogen from glucose-1-phosphate, the explanation seems to be that glucose-1-phosphate cannot diffuse into the intact muscle and is therefore not used. This is the more probable since it is known that glucose phosphates are only formed inside the cells and do not diffuse in or out of them.

DISCUSSION

We have thus confirmed the finding of Gemmill (Gemmill, 1940, 1941; Gemmill & Hamman, 1941) and of Soskin (Hechter *et al.* 1941) that the surviving diaphragm of rats can produce glycogen from glucose in the presence of insulin. We were also able to show that, contrary to the above authors, even without insulin glycogen can be formed in a solution containing not more than 100 mg. glucose/100 ml. Insulin increases this glycogen production three or four times. Whilst the American authors used 100–200 units insulin/100 ml., we found that even with 0·5–1 unit/100 ml. an almost maximal glycogen production was obtained. The glycogen production occurs mainly in the first half hour and slows down afterwards.

Diaphragms of adrenalectomized rats produced glycogen in the same amounts as those of normal animals. Thus the specific disturbance in adrenal cortical insufficiency cannot be an inability to produce muscle glycogen from glucose.

It was also shown that the addition of DOC with glucose alone, or with glucose and insulin, totally inhibited glycogen formation. The hormone was active in quantities of 1–10 mg./100 g. muscle, or for a diaphragm of 100 mg. in quantities of 1–10 μg. About 1 mg. DOC daily would be needed to keep alive an adrenalectomized rat of 150 g. body weight with 50 g. muscle tissue; this corresponds to about 2 mg./100 g. muscle. Thus the concentrations which were effective in these experiments *in vitro* are equivalent to the doses needed to keep an adrenalectomized animal alive.

In these experiments, therefore, DOC acts by increasing the breakdown of glycogen (glycogenolysis). This is in accordance with the fact previously announced in several papers from this laboratory (Verzár & Montigel, 1942, 1943*a*, 1943*b*; Doetsch, 1945), that after adrenalectomy the phosphorylation of glycogen by muscle (and liver) decreases. Glycogen-phosphorolysis is the first step in glycogen breakdown to form glucose-1-phosphate. As was shown by the above authors, DOC acts by accelerating this process.

Since however it is known that the same enzyme (phosphorylase) can also catalyze the synthesis of glycogen (Cori *et al.* 1939; Cori, Green & Cori, 1942; Kiessling, 1939; Ostern & Holmes, 1939), it has been thought until now that the disturbance might be on the side of glycogenesis rather than of glycogeno-

lysis. The experiments in this paper show that the disturbance in the undamaged muscle lies only on the side of glycogenolysis, which is greatly increased by DOC in normal as well as in adrenalectomized animals.

It is difficult to explain the decrease of glycogen in the organs of adrenalectomized animals. The apparent result of the administration of DOC or other adrenal cortical hormones *in vivo* is an increase of glycogen content, as we ourselves and others have shown (e.g. Verzár & Montigel, 1943*a*, 1943*b*); Koepf *et al.* (1941, 1942) have also shown that in liver slices of adrenalectomized animals which were starved for 24 hr. the so-called total carbohydrate was lower than in normals; and that normal and adrenalectomized animals produced more total carbohydrate from lactate and pyruvate if cortin was previously injected.

In spite of these difficulties, our experiments can only be explained by assuming that DOC increases the glycogenolysis and thus counteracts the effect of insulin, which increases glycogenogenesis.

Since it was shown by Verzár & Wenner (1948), that the minced muscle of adrenalectomized animals can produce glycogen from Cori ester, while the undamaged diaphragm cannot, it seems that glucose-1-phosphate cannot diffuse into the undamaged muscle fibres (Conway, 1942). On the other hand, it can be used for glycogen formation in minced muscle from adrenalectomized as well as from normal animals. Thus the disturbance which leads to a decrease of phosphorylation of glycogen after adrenalectomy is on the breakdown side of glycogen metabolism: it is the first step of glycogen breakdown (glycogenolysis), the phosphorylation of glycogen in muscle, which is decreased after adrenalectomy. This process is increased in the whole muscle by DOC in normal and adrenalectomized animals. The increase of glycogenolysis through DOC can totally antagonize the action of insulin upon glycogen synthesis in muscle.

Further experiments with other steroids which we propose to do, are intended to show whether this effect which we have found is specific for DOC.

SUMMARY

1. The surviving diaphragm of the rat, in Ringer solution saturated with oxygen and buffered with phosphate, synthesizes glycogen when 100 mg. glucose/100 ml. are added. In pure Ringer solution, it breaks down its own glycogen stores.

2. The addition of 0·5–1 unit of insulin/100 ml. to the Ringer solution containing 100 mg. glucose/100 ml., increases the formation of glycogen. A further addition of insulin, up to 100 units/100 ml., produces no further increase in glycogen formation.

3. After the addition of 5–10 mg. of DOC/100 ml. to the Ringer solution there is a breakdown of glycogen in the diaphragm. When the Ringer solution contains glucose, the synthesis of glycogen is completely inhibited by the addition of even 1 mg. DOC/100 ml. When 1 unit of insulin/100 ml. is added to the Ringer solution with glucose, then 5–10 mg. DOC/100 ml. completely prevent the synthesis of glycogen. With 1 mg. DOC/100 ml. scarcely any action could be detected. Even when 100 units of insulin/100 ml. are added, no synthesis occurs in the presence of 10 mg. DOC/100 ml.

4. The muscles of adrenalectomized animals synthesize glycogen to the same extent as those of normal animals, and in these animals also DOC favours the breakdown of glycogen and inhibits the action of insulin.

5. If glucose-1-phosphate is taken instead of glucose then no synthesis of glycogen occurs even in the presence of insulin. The explanation may be that the cell membrane is non-permeable to this ester.

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REFERENCES

Conway, E. J. (1942). *Nature, Lond.*, **150**, 461.
Cori, C. F., Colowick, S. P. & Cori, G. T. (1937). *J. biol. Chem.* **121**, 465.
Cori, G. T., Green, A. A. & Cori, C. F. (1942). *J. biol. Chem.* **142**, 447.
Cori, C. F., Schmidt, G. & Cori, G. T. (1939). *Science*, **89**, 464.
Doetsch, R. (1945). *Helv. chim. Acta*, **28**, 31.
Gemmill, C. L. (1940). *Johns Hopk. Hosp. Bull.* **66**, 232.
Gemmill, C. L. (1941). *Johns Hopk. Hosp. Bull.* **68**, 329.
Gemmill, C. L. & Hamman, L. (1941). *Johns Hopk. Hosp. Bull.* **68**, 50.
Hallmann, L. (1939). *Klinische Chemie u. Mikroskopie*, p. 320. Leipzig: Thieme.
Hastings, A. B., Muus, J. & Bessey, O. A. (1939). *J. biol. Chem.* **129**, 295.
Hechter, O., Levine, R. & Soskin, S. (1941). *Proc. Soc. exp. Biol., N.Y.*, **46**, 390.
Kiessling, W. (1939). *Biochem. Z.* **302**, 50.
Koepf, G. F., Horn, H. W., Gemmill, C. L. & Thorn, G. W. (1941). *Amer. J. Physiol.* **133**, Proc. 353.
Koepf, G. F., Horn, H. W., Gemmill, C. L. & Thorn, G. W. (1942). *Amer. J. Physiol.* **135**, 175.
Miescher, K. & Meystre, C. (1943). *Helv. chim. Acta*, **26**, 224.
Ostern, P. & Holmes, E. (1939). *Nature, Lond.*, **144**, 34.
Verzár, F. & Montigel, C. (1942). *Helv. chim. Acta*, **25**, 9, 22.
Verzár, F. & Montigel, C. (1943*a*). *Helv. chim. Acta*, **26**, 883.
Verzár, F. & Montigel, C. (1943*b*). *Helv. physiol. pharmacol. Acta*, **1**, 115, 137.
Verzár, F. & Wenner, V. (1948). *Biochem. J.* **42**, 42.

Glycogen Production from Glucose-1-phosphate by Liver and Muscle of Normal and Adrenalectomized Animals

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It has been shown by Schumann (1940), Verzár & Montigel (1941, 1942, 1943*a*, 1943*b*) and by Kutscher & Wüst (1941, 1942) that, after adrenalectomy, phosphorylation of glycogen by muscle and liver (Doetsch, 1945) is decreased. Since some authors were not able to confirm this observation (Riesser, 1943–5; Smits, 1943–5; Helve, 1940) the experiments were repeated in this laboratory by Staehelin & Voegtli (unpublished) and the earlier results were fully confirmed. The first step in the breakdown of glycogen is the formation of glucose-1-phosphate and, as shown by Cori and his co-workers (Cori, Cori & Hegnauer, 1937; Cori, Colowick & Cori, 1937; Cori, Cori & Schmidt, 1939; Cori, Schmidt & Cori, 1939; Cori & Cori, 1939; Cori, Green & Cori, 1942), this reaction is reversible. The equilibrium mixture at pH 7 consists of 77 % glycogen and 23 % glucose-1-phosphate (Colowick & Sutherland, 1942). Glycogen has been produced *in vitro* with minced tissues (Kiessling, 1936, 1937; Ostern, Herbert & Holmes, 1939; Ostern & Holmes, 1939), tissue extracts (Cori, Cori & Schmidt, 1939; Cori & Cori, 1939; Mirski & Wertheimer, 1942) and with purified or even crystalline enzymes (Cori *et al.* 1937; Cori, Colowick & Cori, 1938; Cori *et al.* 1942; Kiessling, 1939*a*, 1939*b*; Colowick & Sutherland, 1942; Schäffner & Specht, 1938, 1939).

In our former experiments only the breakdown of glycogen was studied. It was therefore of interest to see whether the reverse reaction, the production of glycogen from glucose-1-phosphate, was influenced by adrenalectomy. New experiments of Koepf, Horn, Gemmill & Thorn (1941) and of Verzár & Wenner (1948) had shown that whole muscle of adrenalectomized animals is able to produce glycogen. We have therefore studied the problem as to whether muscle and liver of adrenalectomized animals can produce glycogen from glucose-1-phosphate.

METHODS

White rats of 150–300 g. of the laboratory stock were used. Adrenalectomy was carried out on males only. The animals appeared to be well again 2–3 days after the operation and became adynamic after 5–20, days when they were used for the experiments. Our test for adynamia was as follows: the animal was put on an almost vertical wire netting and its movements were observed. Normal animals always climb upwards, whilst adynamic animals cling on to the netting and fall down after a few minutes. We generally used animals which were able to keep up for at least 0·5 min.

The animals were pithed and then bled. The liver was immediately removed and pressed through a sieve. Muscles of the abdominal wall were cut up in a Latapie mincer. The methods of Ostern *et al.* (1939) and of Ostern & Holmes (1939) were used for the estimation of glycogen production in muscle and liver. Quantities of about 0·5 g. of tissue were quickly weighed on a torsion balance and then put in either (*a*) 1 ml. of 0·8 % NaCl containing 0·005 M-NaF, or (*b*) 1 ml. of a solution containing 0·005 M-NaF and glucose-1-phosphate of a concentration which was equivalent to a glucose content of 10 mg./ml. In certain cases 5 mg. of glycogen (Hofmann-La Roche), dissolved in 0·2 ml. of water, were added to the mixture (i.e. 1 g./100 g. muscle or liver). The muscle or liver mixture was kept at 20° and the glycogen content was measured after 0, 7, 15 and 30 min. Glycogen production usually reached its maximum after 15–30 min. After 1 hr. the newly formed glycogen had disappeared again (Ostern *et al.* 1939; Ostern & Holmes, 1939).

For glycogen estimation (Pflüger, 1909; Simonovits, 1933; Good, Kramer & Somogyi, 1933; Bomskov & Kaulla, 1942; Kiessling, 1939*a*; Cori & Cori, 1939, 1940) 2 ml. of 40 % (w/v) NaOH were added and the mixture was hydrolyzed on a boiling water bath for 15–30 min. The solution was made up with water to 5 ml. and 5 ml. of 96 % (v/v) ethanol were added. The mixture was kept at 0° for 30 min. and the precipitated glycogen was then centrifuged (3000 r.p.m.) for 5 min. Remaining glucose-1-phosphate was extracted by dissolving the precipitate in 1 % NaCl (2 ml.), reprecipitating the glycogen with ethanol (2 ml.) at 0° and centrifuging. The glycogen was finally dried by adding 96 % ethanol (2 ml.), centrifuging and evaporating the ethanol on a water bath. The glycogen was hydrolyzed with 2 N-H_2SO_4 for 2 hr. on a boiling water bath, the solution was transferred to a 50 ml. flask, made alkaline by addition of 2 N-Na_2CO_3 and diluted with water. The glucose was determined by the Hagedorn-Jensen method.

Glucose-1-phosphate was synthesized from glucose according to Cori *et al.* (1937). The preparation contained 80 % of the Ba salt. For the conversion to the Na salt, Ba was precipitated by Na_2SO_4 from a solution acidified with H_2SO_4 and the solution neutralized with 2 N-Na_2CO_3 (litmus).

RESULTS

The glycogen content of minced liver decreases after the death of the animal. To allow for this glycogenolysis, a control in which no glucose-1-phosphate was added was carried out for each experiment. Uncut

Table 1. *Glycogen production by minced normal liver*

Exp. no.	Wt. of rat (g.)	Time (min.)	Glycogen content (mg./100 g.) Without additions	With addition of glucose-1-phosphate	Glycogen produced (mg./100 g.)	Remarks
4	230 ♂	0	4350	—	—	Concentration of glucose-1-phosphate 5%
		15	4300	5000	700	
		30	3890	4750	860	
5	215 ♀	0	6500	—	—	—
		15	6000	6700	700	
		30	5800	6900	1100	
13	250 ♂	0	6190	—	—	—
		7	6010	6440	430	
		15	5870	6470	600	
		30	5730	6020	290	
14	280 ♂	0	3510	—	—	—
		7	3300	3910	610	
		15	3000	3950	950	
		30	3070	4050	980	
15	220 ♀	0	2420	—	—	New glucose-1-phosphate used
		15	770	1870	1100	
		30	520	1880	1360	
	Means	0	4590	—	—	
		15	3990	4800	810 (±90)*	
		30	3800	4720	920 (±180)*	

* Standard error in brackets.

Table 2. *Glycogen production by minced normal muscle*

Exp. no.	Wt. of rat (g.)	Time (min.)	Glycogen content (mg./100 g.) Without additions	With addition of glucose-1-phosphate	Glycogen produced (mg./100 g.)
4	230 ♂	0	690	—	—
		15	480	1060	580
		30	430	780	350
5	215 ♀	0	790	—	—
		15	600	610	10
		30	520	620	100
12	180 ♀	0	510	—	—
		30	350	590	240
13	250 ♂	0	260	—	—
		7	240	390	150
		15	160	280	120
		30	60	140	80
14	280 ♂	0	440	—	—
		7	340	530	190
		15	200	470	270
		30	230	430	200
	Means	0	540	—	—
		15	360	600	240 (±130)*
		30	400	510	110 (±80)*

* Standard error in brackets.

muscle shows only a little glycogenolysis in the first 30 min. after death, but in minced muscle, diluted with 0·8% NaCl, the glycogen content decreases rapidly. Identical glycogen values were obtained with whole muscle, hydrolyzed directly, or with muscle which was first minced and then immediately hydrolyzed by hot NaOH. The glycogen content of different muscles varies widely (Noll & Becker, 1936). We therefore used the muscles of the abdominal wall, because they present a relatively large quantity of

muscle of uniform glycogen content. It was found that the error of glycogen estimation for amounts under 100 mg./100 g. was about 20%, between 100–500 mg./100 g. about 10–20%, and between 500–5000 mg./100 g. it was under 10%.

In all experiments, unless otherwise stated, the amounts of glucose-1-phosphate and of glycogen added were equivalent to a concentration of 3 g. and 1 g./100 g. minced tissue respectively.

1. *Normal fed animals*

Table 1 shows that in all 5 animals minced liver produced glycogen from glucose-1-phosphate. In Exps. 4, 5, 14 and 15 the maximum was reached after 30 min. and in Exp. 13 after 15 min. The greatest glycogen production was seen in Exp. 15 (1360 mg./100 g.). In 0·8% NaCl the glycogen content decreased during the same period from 2420 to 520 mg./100 g.

Whilst, as shown in Table 1, the glycogen content of liver was between 2·4 and 6·5%, the glycogen content of abdominal wall muscle was only 0·26–0·79% (Table 2).

In the five experiments of Table 2, some of which were done on the same animals as in Table 1, addition of glucose-1-phosphate caused the greatest glycogen production in Exp. 4 (580 mg./100 g.) and this was reached after 15 min. Without glucose-1-phosphate the glycogen content decreased from 690 to 480 mg./100 g.

2. *Normal starved animals*

The following experiments were made in order to study the glycogen production by muscle and liver tissue from rats whose glycogen content had been decreased by fasting. Such experiments were required, since in adrenalectomized animals the glycogen content of both liver and muscle is very much decreased, and it appeared possible that this factor might influence glycogen production.

In rats, fasting for 24 hr. reduces the glycogen content of the liver to very low values (FitzGerald, 1938; FitzGerald, Laszt & Verzár, 1938; Verzár, 1939; Kreienberg & Wiesenhüter, 1943), while in muscles the decrease is less (Kreienberg & Wiesenhüter, 1943).

Table 3. *Glycogen production by minced liver from starved animals*

Exp. no.	Wt. of rat (g.)	Time (min.)	Glycogen content (mg./100 g.) Without additions	With addition of glucose-1-phosphate	With addition of glycogen	With addition of glycogen and glucose-1-phosphate	Glycogen produced from glucose-1-phosphate: Without addition of glycogen	With addition of glycogen	Period of starvation (hr.)
6	230 ♂	0	100	—	—	1310	—	—	24
		15	60	80	960	1350	20	390	
		30	80	—	—	1360	—	—	
7	260 ♂	0	70	—	1070	—	—	—	24
		15	20	5	900	970	−15	70	
		30	10	5	880	880	− 5	0	
8	200 ♂	0	—	—	1420	—	—	—	24
		15	—	—	990	1700	—	710	
		30	60	210	810	1350	150	540	
10	200 ♂	0	730	—	—	1600	—	—	24
		15	560	890	1280	1850	330	570	
		30	380	—	1200	1260	—	60	
22	180 ♀	0	40	—	1040	—	—	—	48
		15	20	30	800	1130	10	330	
23	170 ♀	0	30	—	1050	—	—	—	48
		15	20	30	800	1000	10	200	
24	245 ♂	0	160	—	1140	—	—	—	48
		15	90	700	910	1520	610	610	
26	240 ♂	0	180	—	1160	—	—	—	72
		15	50	670	680	1360	620	680	
27	265 ♂	0	30	—	1030	—	—	—	48
		15	20	120	1070	1390	100	320	
25	260 ♂	0	50	—	1070	—	—	—	48
		15	20	610	770	1240	590	470	
28	325 ♂	0	80	—	1130	—	—	—	72
		15	30	310	880	1430	280	550	
	Means	0	150	—	1120	—	—	—	
		15	110	340	920	1360	230 (±80)*	440 (±60)*	

* Standard error in brackets.

Table 4. *Glycogen production by minced muscle from starved animals*

Exp. no.	Wt. of rat (g.)	Time (min.)	Glycogen content (mg./100 g.) Without additions	With addition of glucose-1-phosphate	With addition of glycogen	With addition of glycogen and glucose-1-phosphate	Glycogen produced from glucose-1-phosphate: Without addition of glycogen	With addition of glycogen	Period of starvation (hr.)
8	200 ♂	0	130	—	1140	1110	—	−30	24
		15	—	—	1080	1430	—	350	
		30	60	50	910	1520	−10	610	
9	210 ♂	0	170	110	—	—	−60	—	24
		15	160	180	1280	1460	20	180	
		30	100	—	1200	1450	—	250	
10	200 ♂	0	120	50	1000	1050	−70	50	24
		15	40	90	760	1610	50	850	
		30	50	—	490	1250	—	760	
22	180 ♀	0	40	—	1070	—	—	—	48
		20	10	20	850	1280	10	430	
23	170 ♀	0	30	—	1020	—	—	—	48
		15	20	30	870	1410	10	540	
24	245 ♂	0	60	—	1120	—	—	—	48
		20	80	110	970	1230	30	260	
25	260 ♂	0	40	—	1020	—	—	—	48
		20	10	10	760	1230	0	470	
26	240 ♂	0	40	—	1040	—	—	—	72
		20	30	130	910	1350	100	440	
27	265 ♂	0	30	—	1040	—	—	—	48
		20	30	60	780	1100	30	320	
28	325 ♂	0	30	—	1020	—	—	—	72
		20	20	30	870	1060	10	190	
	Means	0	70	—	1050	—	—	—	
		15	40	70	910	1320	30 (±10)*	410 (±20)*	

* Standard error in brackets.

From Tables 3 and 4 it can be seen that if the glycogen content of liver or muscle is less than 100 mg./100 g., no or almost no production of glycogen from glucose-1-phosphate is observed *in vitro*. Only in Exps. 25 and 28 (Table 3) is glycogen produced in normal quantities by liver, in spite of a low initial glycogen content. Cori & Cori (1940) have shown that the addition of some glycogen to tissue extracts of very low initial glycogen content initiates glycogen formation from glucose-1-phosphate. We therefore added 1 g. glycogen/100 g. liver or muscle in addition to glucose-1-phosphate. This addition of glycogen caused normal quantities of glycogen to be formed, thus confirming Cori's observation.

3. *Adrenalectomized animals*

The animals were killed in severe adynamia. In Tables 5 and 6 + + + + means a state in which the animals would have died in a few hours, while + + + indicates that the rats were still moving but unable to climb, and were falling from a vertical wire netting in 0·5–2 min.

Table 5 shows that the glycogen content of the liver was extremely low and in Exps. 2, 3, 8, 9, 10 and 11 no glycogen was produced by addition of glucose-1-phosphate alone. However, addition of 1 g. glycogen/100 g. liver produced in the same experiments just as much new glycogen as with tissue from normal animals. This glycogen production varied between 150 and 570 mg./100 g. in 15 min. Only in rat no. 7 was the glycogen content of the liver as high as in normal animals, in spite of a distinct adynamia. Without addition of glycogen 470 mg./100 g. of glycogen were produced from glucose-1-phosphate, whilst with addition of glycogen 890 mg./100 g. new glycogen were produced from the phosphate ester. Rat no. 6, in spite of an extremely low glycogen content, produced without addition of glycogen 200 mg./100 g., and with addition of glycogen 400 mg./100 g., of new glycogen from glucose-1-phosphate.

Thus the production of glycogen by tissue from these adrenalectomized animals was not diminished as compared with that with tissue from normal, starved rats. These ten experiments do not indicate any marked effect of adrenalectomy on glycogen

Table 5. *Glycogen production by minced liver from adrenalectomized rats*

Exp. no.	Wt. of rat (g.)	Time (min.)	Glycogen content (mg./100 g.) Without additions	With addition of glucose-1-phosphate	With addition of glycogen	With addition of glycogen and glucose-1-phosphate	Glycogen produced from glucose-1-phosphate: Without addition of glycogen	With addition of glycogen	Days after adrenalectomy, and degree of adynamia
2	195 ♂	0	50	—	—	—	—	—	17
		7	20	10	—	—	−10	—	+ + + +
		15	10	10	—	—	0	—	
		30	20	20	—	—	0	—	
3	190 ♂	0	10	—	—	—	—	—	18
		15	10	5	—	—	− 5	—	+ + +
		30	5	5	—	—	0	—	
4	225 ♂	0	—	—	1220	—	—	—	19
		7	—	—	900	1470	—	570	+ + +
		15	—	—	920	1490	—	570	
5	200 ♂	0	20	—	—	—	—	—	18
		7	—	—	820	1300	—	480	+ + +
		30	—	—	680	1300	—	620	
8	130 ♂	0	30	—	1030	—	—	—	10
		15	10	40	—	1320	30	(320)	+ + +
9	150 ♂	0	20	—	1030	—	—	—	10
		15	30	40	930	1080	10	150	+ + +
10	150 ♂	0	20	—	1020	—	—	—	11
		15	20	10	970	1150	−10	180	+ + + +
11	145 ♂	0	40	—	—	—	—	—	11
		15	30	60	1010	1270	30	260	+ + +
6	117 ♂	0	20	—	1030	—	—	—	5
		15	20	220	830	1230	200	400	+ + +
7	102 ♂	0	2000	—	2900	—	—	—	5
		15	1850	2320	2220	3110	470	890	+ + +
	†Means	0	30	—	1070	—	—	—	
		15	20	60	930	1260	40 (±30)*	330 (±80)*	

* Standard error in brackets.
† Nos. 5 and 7 are not included.

formation from glucose-1-phosphate by liver tissue. Many more experiments would be necessary to reveal possible differences of velocity or slight, statistically significant, differences.

Table 6 shows that muscles of the same adrenalectomized animals too have very low initial glycogen contents. In none of these experiments was there a production of glycogen from glucose-1-phosphate, without addition of extra glycogen. If, however, 1 g. glycogen/100 g. muscle was added, new glycogen was produced in about the same quantity as with starved normal animals. Thus the muscle from adrenalectomized animals too, has not lost the capacity to produce glycogen from glucose-1-phosphate.

DISCUSSION

The above experiments show that normal minced liver and muscle produce glycogen from glucose-1-phosphate. In starved rats with very low glycogen content, however, no glycogen is produced by minced muscle or liver, unless glycogen is added to the solution. This seems to show that the glycogen-producing enzyme (phosphorylase, Cori *et al.* 1942) can only produce glycogen from glucose-1-phosphate if some glycogen is already present. Kiessling (1939*a*, 1939*b*), Cori, Cori & Schmidt (1939) and Colowick & Sutherland (1942) have obtained similar results with pure enzyme solutions. Phosphorylase thus appears to increase the size of glycogen molecules which are already present without increasing their number.

It is well known that adrenalectomized rats show a very low glycogen content in the liver and later also in the muscles. Since starvation for 24–48 hr. in rats produces similarly low glycogen values, it seemed possible that the low glycogen contents of adrenalectomized rats are caused, at least partly, by the concomitant starvation. Liver or muscle tissue from adrenalectomized animals produces no glycogen without addition of extra glycogen and thus behaves like that from starved animals. But glycogen is produced in the same quantities as by tissue from normal animals, if glycogen is added. Thus the disturbance of carbohydrate metabolism

Table 6. *Glycogen production by minced muscle from adrenalectomized animals*

Exp. no.	Wt. of rat (g.)	Time (min.)	Glycogen content (mg./100 g.) Without additions	With addition of glucose-1-phosphate	With addition of glycogen	With addition of glycogen and glucose-1-phosphate	Glycogen produced from glucose-1-phosphate: Without addition of glycogen	With addition of glycogen	Days after adrenalectomy and adynamia
2	195 ♂	0	30	—	—	—	—	—	17
		7	20	—	—	—	—	—	+ + + +
		15	10	10	—	—	0	—	
		30	10	10	—	—	0	—	
3	190 ♂	0	20	—	—	—	—	—	18
		7	10	10	—	—	0	—	+ + +
		15	10	5	—	—	− 5	—	
		30	5	0	—	—	− 5	—	
5	200 ♂	0	40	—	—	—	—	—	18
		7	—	—	1090	1340	—	250	+ + +
		30	—	—	900	1300	—	400	
4	225 ♂	0	—	—	1170	—	—	—	19
		7	—	—	970	1240	—	270	+ + +
		30	—	—	930	1150	—	220	
6	117 ♂	0	30	—	1070	—	—	—	5
		20	—	40	940	1700	(10)	760	+ + +
7	102 ♂	0	80	—	—	—	—	—	5
		20	—	100	—	2240	(50)	(1000)	+ + +
8	130 ♂	0	25	—	—	—	—	—	10
		20	15	25	1000	1420	10	420	+ + +
9	150 ♂	0	110	—	1150	—	—	—	10
		20	20	10	1010	1320	− 10	310	+ + +
10	150 ♂	0	30	—	—	—	—	—	11
		20	20	20	930	1350	0	420	+ + + +
11	145 ♂	0	60	—	—	—	—	—	11
		20	30	70	950	1250	40	300	+ + +
	†Means	0	50	—	—	—	—	—	
		20	20	40	950	1360	20 (±10)*	410 (60)*	

* Standard error in brackets.
† No. 7 is not included.

after adrenalectomy cannot be due to a lack of glycogen production from glucose-1-phosphate. This result is in agreement with the observation with surviving diaphragms of rats (Koepf *et al.* 1941; Verzár & Wenner, 1948) that there is no difference as regards glycogen formation from glucose between diaphragms obtained from normal and adrenalectomized animals. Thus only minced muscle seems to be unable to produce glycogen when the initial glycogen content is low, since undamaged whole muscle retains the ability to produce glycogen under these conditions. The surviving diaphragm produces glycogen from glucose, but not from glucose-1-phosphate (Verzár & Wenner, 1948). This is not incompatible with the present finding that minced liver and muscle produce glycogen from glucose-1-phosphate. The latter, unlike glucose, does not diffuse through cell membranes into the cells of the undamaged surviving diaphragm. Glucose-1-phosphate, like other phosphoric acid esters of glucose, is produced inside the cells. The production of those esters inside the cell is, as pointed out by Verzár & McDougall (1936) and Minibeck & Verzár (1940), the factor responsible for the increased diffusion rate of glucose through cell membranes. In minced liver and muscle most of the cells are damaged. The greater part of the glycogen is not produced inside the cells but by dissolved enzymes in the solution. This may explain the fact that addition of 1% glycogen to the solution increases glycogen production from glucose-1-phosphate. Moreover, it is known that with pure solutions of phosphorylase production of new glycogen takes place only when some glycogen is already present.

The result that muscle and liver tissue from adrenalectomized animals has not lost the ability to form new glycogen is in conformity with the finding that the surviving diaphragm of adrenalectomized rats produces glycogen (Koepf *et al.* 1941; Verzár & Wenner, 1948). The disturbance of carbohydrate metabolism after adrenalectomy is thus due either to an inhibition of phosphorylation, or, more probably, to a disturbance of the breakdown of glycogen and not of its synthesis.

SUMMARY

1. Minced muscle and liver of normal rats produce glycogen from glucose-1-phosphate *in vitro*.

2. If, after a starvation period, the glycogen content falls below 200 mg./100 g., minced liver and muscle do not produce glycogen from glucose-1-phosphate. However, if glycogen is added as well, additional glycogen is produced, just as with pure enzyme solutions.

3. In adrenalectomized, adynamic rats the glycogen content is decreased in liver and muscle, and only if glycogen is added can production of new glycogen by minced muscle and liver be observed to occur at the same rate as with normal animals. Thus adrenalectomy does not affect the mechanism responsible for the synthesis of glycogen from glucose-1-phosphate.

REFERENCES

Bomskov, C. & Kaulla, K. N. (1942). *Z. ges. exp. Med.* **110**, 604.
Colowick, S. P. & Sutherland, W. (1942). *J. biol. Chem.* **144**, 424.
Cori, C. F., Colowick, S. P. & Cori, G. T. (1937). *J. biol. Chem.* **121**, 465.
Cori, G. T., Colowick, S. P. & Cori, C. F. (1938). *J. biol. Chem.* **123**, 375.
Cori, C. F. & Cori, G. T. (1939). *J. biol. Chem.* **131**, 397.
Cori, G. T. & Cori, C. F. (1940). *J. biol. Chem.* **135**, 733.
Cori, C. F., Cori, G. T. & Hegnauer, A. H. (1937). *J. biol. Chem.* **120**, 193.
Cori, G. T., Cori, C. F. & Schmidt, G. (1939). *J. biol. Chem.* **129**, 629.
Cori, C. F., Green, A. A. & Cori, G. T. (1942). *J. biol. Chem.* **142**, 447.
Cori, C. F., Schmidt, G. & Cori, G. T. (1939). *Science*, **89**, 464.
Doetsch, R. (1945). *Helv. chim. Acta*, **28**, 31.
FitzGerald, O. (1938). *Verh. Ver. Schweiz. Physiol.* **13**, 14.
FitzGerald, O., Laszt, L. & Verzár, F. (1938). *Pflüg. Arch. ges. Physiol.* **240**, 619.
Good, C. A., Kramer, H. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 485.
Helve, O. E. (1940). *Biochem. Z.* **306**, 358.
Kiessling, W. (1936). *Biochem. Z.* **287**, 291.
Kiessling, W. (1937). *Biochem. Z.* **292**, 26.
Kiessling, W. (1939*a*). *Biochem. Z.* **302**, 50.
Kiessling, W. (1939*b*). *Naturwissenschaften*, **27**, 129.
Koepf, G. F., Horn, H. W., Gemmill, C. L. & Thorn, G. W. (1941). *Amer. J. Physiol.* **133**, Proc. 353.
Kreienberg, W. & Wiesenhüter, E. (1943). *Pflüg. Arch. ges. Physiol.* **247**, 22.
Kutscher, W. & Wüst, H. (1941). *Naturwissenschaften*, **29**, 314.
Kutscher, W. & Wüst, H. (1942). *Hoppe-Seyl. Z.* **273**, 235.
Minibeck, H. & Verzár, F. (1940). *Helv. med. Acta*, **7**, Suppl. v, 7.
Mirski, A. & Wertheimer, E. (1942). *Biochem. J.* **36**, 230.
Noll, A. & Becker, M. (1936). *Biochem. Z.* **287**, 88.
Ostern, P., Herbert, D. & Holmes, E. (1939). *Biochem. J.* **33**, 1858.
Ostern, P. & Holmes, E. (1939). *Nature, Lond.*, **144**, 34.
Pflüger, E. (1909). *Pflüg. Arch. ges. Physiol.* **129**, 362.
Riesser, O. (1943–5). *Enzymologia*, **11**, 323.
Schäffner, A. & Specht, H. (1938). *Naturwissenschaften*, **26**, 494.
Schäffner, A. & Specht, H. (1939). *Naturwissenschaften*, **27**, 195.
Schumann, H. (1940). *Pflüg. Arch. ges. Physiol.* **243**, 686.
Simonovits, S. (1933). *Biochem. Z.* **265**, 437.
Smits, G. (1943–5). *Enzymologia*, **11**, 334.
Verzár, F. (1939). *Die Funktion der Nebennierenrinde*, p. 62. Basel: Schwabe.
Verzár, F. & McDougall, E. J. (1936). *Absorption from the Intestine*. London: Longmans, Green and Co. Ltd.
Verzár, F. & Montigel, C. (1941). *Schweiz. med. Wschr.* **71**, 101.
Verzár, F. & Montigel, C. (1942). *Helv. chim. Acta*, **25**, 9, 22.
Verzár, F. & Montigel, C. (1943*a*). *Helv. chim. Acta*, **26**, 883.
Verzár, F. & Montigel, C. (1943*b*). *Helv. physiol. pharmacol. Acta*, **1**, 115, 137.
Verzár, F. & Wenner, V. (1948). *Biochem. J.* **42**, 35.

The Action of Steroids on Glycogen Breakdown in Surviving Muscle

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In a previous paper (Verzár & Wenner, 1948) the effect of deoxycorticosterone (DOC) on glycogen synthesis from glucose with or without insulin was studied in the surviving diaphragm of the rat. DOC in concentrations of 5 mg./100 ml. of suspending fluid counteracts the effect of insulin and increases glycogen breakdown. In order to establish whether this effect of DOC is specific, several other steroids, sex hormones and similar biologically active and inactive substances have been tested in the same way.

METHODS

The technique used was that of Gemmill (1940, 1941) and of Gemmill & Hamman (1941) with modifications as described in our previous paper (Verzár & Wenner, 1948). The diaphragms of rats were incubated in oxygenated,

phosphate buffer Ringer solution containing 100 mg. glucose and 1 unit insulin/100 ml. of solution. The steroid was added in a 2% (w/v) solution in ethanol or in a 1% solution in 50% (v/v) ethanol, if this was possible without crystallization occurring. Thus 0·05 or 0·1 ml. had to be added to 10 ml. solution to give a concentration of 5 or 10 mg. steroid/100 ml. solution.

Surface tension was measured with the Traube stalagmometer (Traube, 1887; Abderhalden, 1930). For these experiments the 50% ethanolic solutions of the steroids were diluted with distilled water to a concentration of 5 or 10 mg./100 ml. The surface tension is expressed in dynes/cm. (Landolt-Börnstein, 1923).

RESULTS

Table 1 shows the effect of various steroids on glycogen breakdown. Each line contains the mean values of a series of at least 4, but mostly of 8 or more single experiments. Steroids 1–14 contain either free keto or hydroxyl groups or both, whilst steroids 15–17 contain either an ether or an ester linkage. Substances 18 and 19 are not related to steroids. The effect on glycogen breakdown is seen from a comparison of columns 2 and 3. The dose of 5 mg./100 ml. was chosen to show the different effects of the steroids. DOC in a concentration of 5 mg./100 ml. of solution inhibits glycogen production in the presence of 100 mg. sugar and 1 unit insulin/100 ml. solution. Progesterone (no. 2) and pregnenediolone (no. 4) show even a larger effect. With 5 mg. progesterone the glycogen content is decreased even below the initial value.

Pregnanediolone, corticosterone, hydroxycorticosterone, testosterone, androstanediol (nos. 5, 6, 7, 8, 9) have a distinctly smaller effect. 11-Dehydrocorticosterone (no. 17) was also active; it could only be tested as acetate. Pregnanediolone, testosterone and androstanediol were, in the higher concentration (10 mg./100 ml.), as active as DOC; but oestradiol (no. 10) and bisdehydrodoisynolic acid (no. 11) had the same activity at a concentration of 10 mg./100 ml. as the former group (nos. 5–9) had at a concentration of 5 mg./100 ml. Hydroxyaetiocholenic acid (no. 12), *allo*pregnanepentol (no. 13), and androstenediol (no. 14) had no action even in the higher concentration of 10 mg./100 ml. Stilboestrol (no. 18), an oestrogen, was more active than oestradiol and its activity was equal to that of progesterone or DOC.

The glucoside of DOC (no. 15) had no activity, while the monoacetate of pregnenediolone (no. 16) had about the same activity as the non-esterified substance (no. 4). The acetate of 11-dehydrocorticosterone was also very active. Oleic acid (no. 19), with a molecular weight similar to those of the steroids tested, was without activity.

DISCUSSION

The inhibition of glycogen production and increase of glycogen breakdown is not present in substances lacking a hormone-like action such as hydroxyaetiocholenic acid and oleic acid. On the other hand high activity was found in stilboestrol and, to a lesser extent, in bisdehydrodoisynolic acid, substances which are chemically different and only related biologically to the steroid hormones.

In the DOC-21-glucoside (no. 15) the new linkage on the 21-hydroxyl group abolished the whole activity. It is possible that this substance cannot be split by intact muscle, but the acetate of pregnenediolone (no. 16) was as active as pregnenediolone itself. The acetate of 11-dehydrocorticosterone (no. 17) was also active. Thus, if the above explanation is correct, muscle must be able to split these two esters. It should be remembered that in previous experiments on glycogen phosphorylation (Montigel & Verzár, 1943) it was found that steroids esterified in the 21-position were less active.

It is much more difficult to explain the differences between the activities of the various other substances on glycogen breakdown. It might appear that the 11-hydroxysteroids which are more soluble in water have a smaller activity. Thus the most active substances progesterone (no. 2), pregnenediolone (no. 4) and stilboestrol (no. 18), which are active even in a concentration of 1 mg./100 ml., have a smaller solubility in water than corticosterone (no. 6) and *allo*pregnanepentol (no. 13), which are less active. On the other hand oestradiol (no. 10) and androstenediol (no. 14) are less soluble in water than progesterone (no. 2), but have a smaller activity than corticosterone (no. 6). Thus differences in water solubility do not explain the greater activity of certain steroids on the inhibition of glycogen formation.

Another explanation might be found in the surface activity of steroids (Sobotka, 1938). The steroids, after adsorption on the cell surface might inhibit the diffusion of glucose or of oxygen into the cells. The substances most active in glycogen breakdown, such as DOC and pregnenediolone, lower the surface tension of water to 68 and 68·5 (dynes/cm.) respectively; the inactive oestradiol had in aqueous solution a surface tension of 70; and the fairly active corticosterone 58·5. Thus there was no correlation between inhibition of glycogen formation and surface tension.

There is a marked difference in inhibitory action on glycogen production between DOC and progesterone, both of which are very active, and corticosterone and 17-hydroxycorticosterone which show very little activity. The second group also has a distinct action on glycogen production in the whole animal and this is actually used in some assays of hormones of the adrenal cortex, such as the work test of Ingle (1941) and the glycogen deposition test (Reinecke & Kendall, 1942; Dorfman, Ross & Shipley, 1946; Eggleston, Johnston & Dobriner,

1946). Many authors therefore believe that 11-hydroxysteroids have a specific effect on carbohydrate metabolism, whilst DOC has none. This point has been discussed by us (Verzár, Bucher, Somogyi & Wirz, 1941), and we consider that there is a difference in the velocity but not in the nature of the action of the two groups of steroids. It is remarkable that the results described in this paper show differences between these two groups also with respect to glycogen metabolism *in vitro*.

It has been known for a long time that the sex hormones show effects on carbohydrate metabolism which are comparable with those of the adrenal cortical hormones. Montigel & Verzár (1943) have shown that progesterone, testosterone and to a smaller degree oestradiol influence phosphorylation of glycogen by muscle. The activity is of the same order as that observed in the present experiments. Ingle (1940, 1943, 1944) and Janes & Nelson (1940) have demonstrated that the naturally occurring sex

Table 1. *The effects of various steroids and related substances on the glycogen content of rat's diaphragm in the presence of glucose and insulin*

	Steroid			Glycogen content (mg./100 g.)			
					After 1·5 hr. with addition of glucose and insulin		
No.	Name	Concentration (mg./100 ml.)	No. of animals	Initial 1	Without steroid 2	With addition of steroid 3	Mean difference (mg./100 g.) 3–2	S.E.± (mg./100 g.)
1	Deoxycorticosterone	10	21	213	309	156	−153	18
		5	30	246	380	244	−136	13
		1	20	287	422	402	− 20	24
2	Progesterone	10	12	325	410	237	−173	27
		5	8	359	456	252	−204	30
		1	7	283	400	308	− 92	36
3	17-Hydroxyprogesterone	10	7	209	443	241	−202	53
4	Pregn-5-ene-3(β):21-diol-20-one	10	4	275	405	212	−193	20
		5	4	290	475	305	−170	20
		1	12	287	415	368	− 47	14
5	Pregnane-3(α):12(β)-diol-20-one	10	8	305	412	285	−127	22
		5	8	356	455	392	− 63	21
6	Corticosterone	5	16	339	433	367	− 66	16
7	17-Hydroxycorticosterone	5	8	331	485	441	− 44	27
		1	8	260	365	355	− 10	37
8	Testosterone	10	8	405	536	292	−244	20
		5	8	281	384	312	− 72	25
		1	8	200	360	228	−132	36
9	Androstane-3(β):17-diol	10	6	423	527	395	−132	52
		5	12	257	389	320	− 69	18
10	Oestradiol	10	8	201	360	278	− 82	15
		5	8	190	344	334	− 10	24
11	Bisdehydrodoisynolic acid	10	8	254	420	310	−110	18
		5	8	192	351	355	+ 4	22
12	3(β)-Hydroxyaetiochol-5-enic acid	10	8	309	366	381	+ 15	18
13	*allo*Pregnane-3(β):11(β):17:20:21-pentol	10	7	285	360	321	− 39	33
14	Androst-5-ene-3(β):17-diol	10	8	280	428	400	− 28	35
		5	8	269	421	385	− 36	13
15	Deoxycorticosterone-β-*d*-glucoside	10*	20	252	364	336	− 28	26
16	Pregn-5-ene-3(β):21-diol-20-one-21-acetate	10	7	237	351	214	−137	28
17	11-Dehydrocorticosterone-acetate	10	8	340	462	370	− 92	24
18	Stilboestrol (4:4′-dihydroxy-αβ-diethylstilbene)	10	6	202	302	148	−154	37
		5	8	199	348	204	−144	27
		1	10	206	310	252	− 58	28
19	Oleic acid (Na salt)	10	4	438	655	790	+ 45	33
		5	4	358	562	594	+ 35	34

* Calculated as pure DOC.

hormones and stilboestrol have a marked influence on the sugar excretion of pancreatectomized rats. It has also been shown (Kochakian, 1945–6) that testosterone lowers the activity of the alkaline and enhances the activity of the acid phosphatase of the kidney. Androstanediol has an effect on phosphatases similar to that of testosterone, but androstenediol has no effect.

In our present experiments on glycogen breakdown we found that testosterone (no. 8) was very active, androstanediol (no. 9) less active, while androstenediol (no. 14) had no activity. It is probable that the effect on glycogen breakdown in our experiments, which is especially marked with progesterone and weak with oestradiol, has its parallel in the action of these substances on phosphorylation of glycogen. Thus the experimental results of the inhibition of insulin activity and stimulation of glycogen breakdown in isolated muscle, shown here with DOC and some sex hormones, has its parallel in many other facts. This is not an argument against the specificity of the action of the adrenal cortical hormones on carbohydrate metabolism. Sex hormones appear to act specifically on certain tissues of secondary sexual character, and their main metabolic function might be of the same nature, as was pointed out long ago by one of us. It might be mentioned that we observed that the insulin effect on glycogen synthesis changes throughout the year, which may be connected with varying sex-hormone production in the animals.

It has not been possible to explain how the steroid hormones act on cell metabolism (Long, 1942; Ingle, 1945). It has been suggested that they are 'velocity factors' acting on enzymic reactions of carbohydrate metabolism (Montigel & Verzár, 1943). Another possibility is that the steroids are adsorbed on the surface of the cells and inhibit diffusion of oxygen so that an anaerobic breakdown of glycogen would result. Alternatively, it is possible that the diffusion of glucose from the solution into muscle fibres may be inhibited by the adsorption of the steroids on the cell surface. Whilst such possibilities cannot be denied, an action on intracellular enzymes active in the intermediary carbohydrate metabolism still seems the most probable, although unproven, possibility.

SUMMARY

1. The effect of deoxycorticosterone (DOC) on glycogen breakdown in the intact diaphragm of the rat *in vitro* has been compared with the effect of several other steroid hormones and related substances.

2. Progesterone, 17-hydroxyprogesterone and pregnenediolone had an equal or even stronger effect. Corticosterone, 17-hydroxycorticosterone, testosterone, androstanediol and pregnanediolone showed a smaller effect. Oestradiol, bisdehydroisynolic acid, 3-hydroxyaetiocholenic acid, *allo*-pregnanepentol and androstenediol had the smallest activity, whilst stilboestrol showed a very high activity.

3. The steroids with corticoid activity possessing a hydroxyl group in the 11-position were less active than DOC. The biological activities of the steroids tested did not go parallel with their water solubilities, nor did differences in surface activity explain the differing activities of these substances on glycogen breakdown and the inhibition of the effect of insulin on glycogen production. There is, however, a certain parallelism between the activities of these substances towards glycogen breakdown as shown in the present experiments and their effects on glycogen phosphorylation by minced muscle.

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REFERENCES

Abderhalden, E. (1930). *Hand. Biol. ArbMeth.* Abt. III, Teil A, **2**, p. 1554.
Dorfman, R. J., Ross, E. & Shipley, R. A. (1946). *Endocrinology*, **38**, 178.
Eggleston, N. M., Johnston, B. J. & Dobriner, K. (1946). *Endocrinology*, **38**, 197.
Gemmill, C. L. (1940). *Johns Hopk. Hosp. Bull.* **66**, 232.
Gemmill, C. L. (1941). *Johns Hopk. Hosp. Bull.* **68**, 329.
Gemmill, C. L. & Hamman, L. (1941). *Johns Hopk. Hosp. Bull.* **68**, 50.
Ingle, D. J. (1940). *Proc. Soc. exp. Biol., N.Y.*, **44**, 176.
Ingle, D. J. (1941). *Amer. J. Physiol.* **133**, 676.
Ingle, D. J. (1943). *Endocrinology*, **29**, 838.
Ingle, D. J. (1944). *Endocrinology*, **34**, 361.
Ingle, D. J. (1945). *Ann. Rev. Physiol.* **7**, 527.
Janes, R. G. & Nelson, W. O. (1940). *Proc. Soc. exp. Biol., N.Y.*, **43**, 340.
Kochakian, C. D. (1945–6). *Amer. J. Physiol.* **145**, 118.
Landolt-Börnstein (1923). *Physikalisch-Chemische Tabellen*, 5th ed. part I, pp. 198, 238. Berlin: Springer.
Long, C. N. H. (1942). *Endocrinology*, **30**, 870.
Montigel, C. & Verzár, F. (1943). *Helv. physiol. Acta*, **1**, 115.
Reinecke, R. M. & Kendall, E. C. (1942). *Endocrinology*, **31**, 573.
Sobotka, H. (1938). *The Chemistry of the Sterids*, p. 146. Baltimore: Williams and Wilkins.
Traube, J. (1887). *Ber. dtsch. chem. Ges.* **20**, 2644.
Verzár, F., Bucher, R., Somogyi, J. C. & Wirz, H. (1941). *Helv. med. Acta*, **7**, VI, 58.
Verzár, F. & Wenner, V. (1948). *Biochem. J.* **42**, 35.

A New Method for the Determination of 1:2-Dithiols

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The use of 1:2-dimercaptopropanol (BAL) for the treatment of systemic arsenic intoxication after massive contamination with arsenical vesicants, and after overdosage with arsenicals during arsenotherapy, makes essential a method of high sensitivity for its determination, especially in biological materials. The only method at present available (Spray, 1944) depends on the colour produced by the addition of cobalt salts to solutions of BAL. This method is rather insensitive and the metallic complex, being insoluble, has to be maintained in solution by the use of a protective colloid, a procedure which gives uncertain results. It is also by no means specific, for cobalt salts give colours with almost all thio compounds (Spray, Stocken & Thompson, 1945). The procedure adopted by Spray (1944) for determination in blood involved ultrafiltration after acidification to stabilize the BAL; by this means 80–85% of BAL was recovered from plasma and some 40–60% from blood. In our opinion any method which depends on the isolation of BAL as such from blood is likely to have little success, it being too unstable in this medium.

EXPERIMENTAL

Principles of method

It has been found that 1:2-dithiols react with cyanogen chloride to produce an unidentified compound which readily liberates thiocyanate in alkaline solution. The determination of thiocyanate in micro-quantities has been previously reported (Aldridge, 1944, 1945).

For its determination in biological materials, we have not attempted the isolation of BAL but have converted it to the much more stable compound of BAL with cyanogen chloride and then recovered this compound by a protein precipitation technique (Folin, 1930).

Reagents

Cyanogen chloride solution. (*a*) For determination of BAL in aqueous solution carefully pipette 0·1 ml. (approx.) cyanogen chloride, using an ice-cold pipette, into 200 ml. of Na acetate (*c.* 1 N). (*b*) For determination of BAL in blood and urine, 0·1 ml. cyanogen chloride in 100 ml. 3·8% (w/v) $Na_2SO_4.10H_2O$. Great care should be exercised in the handling of cyanogen chloride. It is highly toxic and extremely volatile (b.p. 14°) and should be stored in a refrigerator in a bottle with a well greased stopper having an external joint.

Bromine water. (*a*) For determination of BAL in aqueous solution, a half-saturated solution. (*b*) For determination of BAL in blood and urine, saturated solution.

Arsenious acid. 2% (w/v) solution. To obtain complete solution boil under reflux.

N-Sodium acetate. 13·6% (w/v) solution of sodium acetate, $CH_3.COONa.2H_2O$.

Sodium tungstate. 10% (w/v) solution $Na_2WO_4.2H_2O$ neutralized to phenolphthalein.

Pyridine reagent. To 1 l. of the constant boiling mixture of pyridine and water (59% pyridine), add 100 ml. of conc. HCl.

Benzidine reagent. 5% (w/v) solution of recrystallized benzidine hydrochloride in 2% (v/v) HCl.

Acids, etc. N-NaOH, 3 N-HCl, 0·66 N-H_2SO_4.

Reaction of dithiols with cyanogen chloride

The reaction of cyanogen chloride with 1:2-dithiols in aqueous solution has been studied at pH values of 6–10. To 6 ml. 0·05 M-phosphate buffer at the required pH (checked by glass electrode) were added 2 ml. cyanogen chloride solution (1 mg.) and 2 ml. BAL solution (70 μg.). At various times, 1 ml. of the above solution was pipetted into 1·8 ml. 0·2 N-NaOH. After leaving for 10 min., the solution was rendered acid by the addition of 0·2 ml. 3 N-HCl. The thiocyanate formed was estimated in the usual way (Aldridge, 1944, 1945). It is seen from Table 1 that the reaction is complete in 2 min. at pH 7–8 while at pH 6 it requires 15 min. As a convenient acidity for the reaction, pH 7 has been chosen, though any high pH may be used provided sufficient excess of cyanogen chloride is added in order that the reaction between BAL and cyanogen chloride is complete before all the cyanogen chloride is hydrolyzed. The poor stability of BAL at high pH prevents the use of very alkaline solutions.

Table 1. *Speed of reaction of cyanogen chloride with 1:2-dithiols at various acidities*

(Thiocyanate determined at various times.)

Time (min.)	Spekker readings		
	pH 6·0	pH 7·0	pH 8·0
0·5	0·36	0·50	0·495
1	0·41	0·51	0·505
2	0·44	0·54	0·54
4	0·465	0·54	0·54
8	0·52	0·54	0·54
16	0·535	—	—

Cyanogen chloride may be determined in aqueous solution by omitting the bromination stage in the normal thiocyanate method. By a determination of the residual cyanogen chloride after reaction with BAL at various pH, it has been possible to determine the molecular proportions of cyanogen chloride and BAL taking part in the reaction. Within the limits of experimental error, 1 mol. of cyanogen chloride reacts with 1 mol. of dithiol (Table 2).

Table 2. *Determination of molecular proportions of cyanogen chloride and BAL in reaction*

(34 μg. BAL taken and excess cyanogen chloride determined after completion of reaction at various pH. Blank contains all reagents except dithiol.)

pH	Spekker reading		Cyanogen chloride reacting (μg.)	Mol. cyanogen chloride /mol. BAL
	Blank	Dithiol		
6·32	1·04	0·26	15·9	0·93
6·94	1·04	0·25	16·1	0·94
7·96	1·03	0·20	16·9	0·99
9·95	0·92	0·15	15·7	0·92

As will be shown later, 1:3-dimercaptopropanol does not interfere in the estimation but acts as a reducing agent and cyanogen chloride is converted to cyanide and chloride. The cyanogen chloride which reacts with 1:3-dithiol is determined as given previously, and cyanogen chloride together with HCN is estimated by including the bromination process as for thiocyanate estimation. The results (Table 3) show that all the reacting cyanogen chloride is reduced to cyanide. Examination of BAL in an identical manner indicates that of the 1 mol. of cyanogen chloride reacting with 1 mol. BAL, 0·05 mol. cyanide is produced (Table 3).

Estimation of SH groups present during the reaction by titration in acid solution with iodine shows that after the initial reaction with cyanogen chloride both SH groups are substituted, and that after alkaline treatment to liberate thiocyanate from the compound of BAL with cyanogen chloride, some indication of the reappearance of SH groups is shown (Table 4). The fact that SH groups are present after treatment with NaOH, in which medium dithiols are rapidly oxidized to the disulphide form, is a strong indication that a greater proportion was produced than that titrated.

Alkaline treatment of the compound of BAL with cyanogen chloride

The reaction product between cyanogen chloride and 1:2-dithiols, although stable in acid solution, liberates thiocyanate completely 5 min. after making alkaline with NaOH. Thiocyanate is also liberated, though more slowly, at pH values 9–10 (Table 5).

The excess cyanogen chloride left in solution is also destroyed by treatment with alkali, being in the main converted to sodium cyanate. However, a small percentage is converted to cyanide (Aldridge, 1945) which would of course react in the same manner as thiocyanate in the subsequent determination. The cyanide is readily removed by making the solution acid after alkaline treatment for 10 min. and bubbling air through the solution for 30 min. (air saturated with water vapour is used to prevent evaporation of the solution). This has no effect on the thiocyanate (Aldridge, 1944). With a blank determination, adding all the reagents except the dithiol, a zero reading on the Spekker absorptiometer is obtained with Ilford spectrum green 604 filters.

It has been shown that a small proportion of cyanide is liberated together with thiocyanate during the alkaline treatment. To determine the amount of cyanide, solutions of the BAL-cyanogen chloride compound were rendered acid and aerated to remove excess cyanogen chloride. Thiocyanate and cyanide were then estimated by the normal procedure and thiocyanate alone after removal of the cyanide by further aeration. Table 6 shows that while about 0·8 mol. thiocyanate are liberated from 1 mol. BAL, 0·05 mol. cyanide is also produced.

It should be noted that after the reaction of 1:2-dithiols with cyanogen chloride there are two possible procedures: (*a*) render the solution acid and aerate to remove excess cyanogen chloride, before liberating the thiocyanate, or (*b*) remove cyanide produced during destruction of excess

Table 3. *Reaction of* 1:3-*dimercaptopropanol and BAL with cyanogen chloride at* pH 7

(Cyanogen chloride and cyanogen chloride + HCN determined after completion of reaction. Blank contains all reagents except dithiol.)

Estimation	Spekker readings		
	Blank	Dithiol	
	1:3-Dimercaptopropanol		
CNCl	0·625	0·030	CNCl reacting, 0·98 mol./mol. dithiol
CNCl + HCN	0·630	0·630	—
HCN (by diff.)	0·005	0·600	HCN produced, 0·98 mol./mol. dithiol
	1:2-Dimercaptopropanol (BAL)		
CNCl	0·980	0·290	CNCl reacting, 1·01 mol./mol. dithiol
CNCl + HCN	1·010	0·355	—
HCN (by diff.)	0·030	0·065	HCN produced, 0·052 mol./mol. dithiol

Table 4. *Determination of SH groups during BAL-cyanogen chloride reaction*

(SH groups titrated with 0·01 N-I_2 in acid solution.)

Time of estimation	Titration (ml. 0·01 N-I_2)	Equivalent SH groups
Initial	1·58	1·96
After reaction with CNCl	0·02	—
After alkaline treatment	0·16	

Table 5. *Liberation of thiocyanate from BAL-cyanogen chloride compound at various acidities at room temperature*

Caustic alkali			pH 9			pH 10·0		
Time (min.)	Spekker reading	% of total	Time (min.)	Spekker reading	% of total	Time (min.)	Spekker reading	% of total
1	0·390	93	3	0·037	8·5	3	0·110	25
2	0·400	95	6	0·052	12	6	0·130	29·5
4	0·405	96	12	0·065	15	12	0·185	42
10	0·410	98	24	0·080	18·5	24	0·255	58
16	0·410	98	48	0·112	26	48	0·315	72
24 hr.	0·420	100						

Table 6. *Alkaline treatment of BAL-cyanogen chloride compound*

(Excess cyanogen chloride removed by aeration in acid solution followed by alkaline treatment. Thiocyanate + cyanide and thiocyanate estimated.)

Estimation	Spekker readings: Blank	Spekker readings: Dithiol	
HCNS + HCN	0·052	0·790	—
HCNS	0·020	0·720	HCNS produced, 0·79 mol./mol. BAL
HCN (by diff.)	0·030	0·070	HCN produced, 0·05 mol./mol. BAL

cyanogen chloride with alkali as given in the description of the method. Procedure (*a*) is useful in the elimination of interference of ergothioneine and gives slightly higher results than (*b*) owing to the extra 0·05 mol. cyanide/mol. BAL (Table 6). Procedure (*b*) has been adopted because the removal of the relatively large quantity of cyanogen chloride by (*a*) requires rather long aeration.

Specificity of the reaction

In order to check the specificity of the reaction for 1:2-dithiols, a large number of compounds has been examined for interference and especially those likely to be encountered in biological fluids. The following is a list of those tested:

1:3-Dimercaptopropanol	Methionine
Hexane-1:6-dithiol	Ethyl sulphide
Monothioethylene glycol	*n*-Butyl sulphide
BAL in disulphide form	Thiophenol
Cysteine hydrochloride	Ethyl mercaptan
Cystine	Ergothioneine
Thiodiglycol	Glutathione
Thioglycollic acid	Thiolacetic acid

The general method we have adopted for the examination of such compounds has been to treat 50 and 30 μg. of the compounds (*c.* 10 μg. of BAL gives a Spekker reading of 1·0) as for the estimation of 1:2-dithiols. In addition, a blank was run with 50 μg. of the compound and all the reagents except cyanogen chloride; this checks for the presence of thiocyanate and any other similar interfering substances. With the above procedure, all except three of the compounds listed in the text gave zero readings: glutathione, ergothioneine and thiolacetic acid. It is not at all clear why glutathione reacts; 40 μg. gives a Spekker reading of 0·09 (*c.* 1 μg. of BAL gives the same reading). It is possible that this result is due to some impurity in the sample of glutathione examined. Ergothioneine reacts slowly with cyanogen chloride at high pH values and to a small extent up to acidities of pH 9 (Table 7). Interference of ergothioneine in the method must therefore take place when NaOH is added to liberate thiocyanate and to destroy excess cyanogen chloride. If necessary, interference from ergothioneine may be prevented by removing excess cyanogen chloride by a stream of air before making alkaline as mentioned previously. However since ergothioneine is not recovered from blood with the protein precipitation procedure to be described, the need for this alteration in the technique is not common. In amounts of 12 μg., thiolacetic acid gives a Spekker reading of 0·45 (Table 8). This is about 25% of the theoretical value expected if 1 mol. of thiocyanate should be obtained from each mol. thiolacetic acid. It is probable that, in the highly alkaline solution, sulphide is produced which reacts with the excess cyanogen chloride or the small amount of cyanide liberated during the hydrolysis of cyanogen chloride.

Table 7. *Speed of reaction of cyanogen chloride with ergothioneine at various acidities*

(Cyanogen chloride allowed to react with ergothioneine for 20 min. Estimation as for that of BAL.)

pH	Spekker reading
7·2	0·02
8·06	0·02
9·0	0·04
10·0	0·150

Table 8. *Interference of thiolacetic acid in the method*

(Various amounts of thiolacetic acid treated as for BAL estimation. Recoveries are based on the theoretical liberation of 1 mol. HCNS/mol. thiolacetic acid.)

Thiolacetic acid (μg.)	Spekker reading	Recovery (%)
6·05	0·220	22·5
12·1	0·455	23·3
18·2	0·605	20·8
24·2	0·835	21·4
30·3	1·03	21·1

Procedure for the estimation of 1:2-dithiols

Description. To 2 ml. of a solution containing not more than 10 μg. of a 1:2-dithiol, add 0·4 ml. of the cyanogen chloride reagent, mix and allow to stand for 5 min. Add 0·4 ml. N-NaOH and allow to stand for 10 min. Make acid by the addition of 0·2 ml. 3N-HCl and bubble air saturated with water vapour through the solution for 30 min. Add 0·2 ml. bromine water, mix and remove excess bromine with 0·2 ml. arsenious acid. Mix well, blow out any bromine vapour left in the tube, and pour into a previously prepared mixture of 4 ml. pyridine reagent and 0·8 ml. benzidine reagent (total volume of solution 8·2 ml.). Mix well by pouring back and forth between the tubes. Allow the colour to develop for 20 min. and then read on the Spekker absorptiometer or any other photoelectric instrument with 1 cm. cells and Ilford spectrum green 604 filters. It should be noted that throughout this work syringe pipettes (maximum error 0·2 %) have been used. With the numerous additions of small quantities of reagents great accuracy in measurements is essential. In order to prevent the catalytic oxidation of the dithiols due to the presence of traces of metals in the distilled water, special precautions have been taken in preparing the standard solutions for the determination of the calibration curves. The solvent used was distilled water, saturated with CO_2, and to which 10 μg. Na cyanide/ml. were added to poison the metallic catalysts. This procedure seems effectively to prevent the oxidation. The figures obtained for the calibration curves for three dithiols are given in Table 9.

Table 9. *Calibration curves for 1:2-dimercaptopropionic acid, 1:2-propane dithiol and BAL*

(Recoveries are based on the theoretical liberation of 1 mol. thiocyanate/mol. of dithiol. Photometric readings (Ilford spectral green 604 filters, 1 cm. cells).)

Amount (μg.)	Spekker reading	Recovery (%)
1:2-Dimercaptopropionic acid		
2·20	0·180	92
4·41	0·350	90
6·61	0·520	89
8·81	0·690	88
11·02	0·860	88
1:2-Propane dithiol		
3·33	0·305	81
6·66	0·610	81
9·99	0·905	80
1:2-Dimercaptopropanol (BAL)		
2·31	0·175	77
4·62	0·550	77
6·93	0·540	79
9·24	0·720	79
11·56	0·905	79

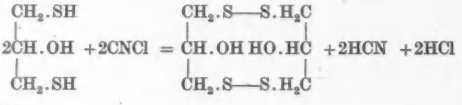
Application to biological fluids. Pipette as soon as possible 1 ml. of whole blood into 1·5 ml. cyanogen chloride reagent in isotonic Na sulphate contained in a centrifuge tube. Leave for 10 min., then add a further 1 ml. cyanogen chloride reagent and leave for 5 min. After this time, add 0·5 ml. 10 % sodium tungstate, mix well and follow by 0·5 ml. 0·66N-H_2SO_4. Mix again and centrifuge for 10 min. The same procedure is satisfactory for BAL in plasma. It is essential to have the blood in contact with the cyanogen chloride as soon as possible to minimize losses of BAL due to decomposition. The procedure used for urine is as given above for blood except that 1 ml. N-sodium acetate is added to the cyanogen chloride reagent before the addition of the urine and no tungstate or sulphuric acid is required. The procedure thenceforward is identical for blood and urine. To not more than 2·0 ml. of filtrate from blood protein precipitation or urine solution add 0·4 ml. N-NaOH and leave for 10 min. Acidify with 0·2 ml. 3N-HCl, make up to 2·6 ml. with water and bubble air saturated with water vapour through the solution for 30 min. to remove cyanide. Brominate with 0·4 ml. bromine water, removing excess with 0·4 ml. arsenious acid. Mix well, blow out any bromine vapour and continue as given under 'description'. For the thiocyanate blank determination, the above procedure is followed replacing the cyanogen chloride reagent with the same volume of isotonic sodium sulphate solution, i.e. 2·5 ml. The difference between the two determinations gives the 1:2-dithiol concentration (plus any interfering substances) and should be referred to the calibration curve as determined before.

DISCUSSION

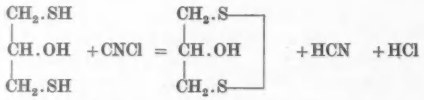
Mechanism of reaction

Of the theoretical yield of thiocyanate based on a reaction of 1 mol. of cyanogen chloride with 1 mol. of BAL, 78 % is obtained (Table 9). Iodine titration of the BAL gave 98·6 % purity. This titration includes substances such as 1:3-dimercaptopropanol but excludes substances such as the oxidized form of BAL. It has been shown that 1 mol. cyanogen chloride reacts with 1 mol. BAL (Table 2) and that 0·8 mol. of thiocyanate is easily liberated in alkaline solution (Table 5); slowly at pH 9 and quickly in NaOH. 1:3-Dimercaptopropanol also reacts with cyanogen chloride but no thiocyanate is liberated in alkaline solution; however, cyanide is liberated equivalent to the cyanogen chloride which has reacted (Table 3). Determination of SH groups during the reaction has shown that both are substituted after the reaction with cyanogen chloride, but after the alkaline treatment they reappear to a small extent (0·2 SH groups/molecule of BAL (Table 4)).

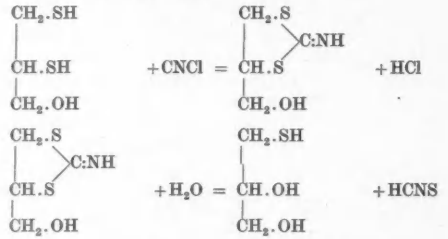
Since cyanide is produced equivalent to the amount of cyanogen chloride reacting with 1:3-dimercaptopropanol, it would seem that it is a simple reduction of cyanogen chloride to cyanide and chloride, i.e.



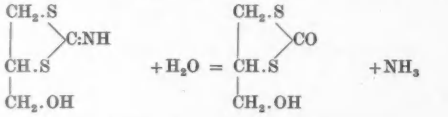
or



Since 1 mol. of cyanogen chloride reacts with 1 mol. of BAL and both SH groups have been shown to be substituted, it is probable that the initial reaction product has a cyclic structure. Further, since thiocyanate is so easily split off, the compound must be unstable and since some indication of the reappearance of SH groups has been obtained, a reaction of the following type may take place:



The compound produced is stable in acid solution and bromination in acid solution without liberating thiocyanate produces no cyanogen bromide. There is also the possibility that an organic thiocyanate is formed, from which no cyanide or thiocyanate would be liberated with the mild conditions in the method. A further possibility is that the cyanogen chloride-BAL compound postulated might be hydrolyzed to produce ammonia, i.e.



The fact that only 0·8 mol. of thiocyanate is obtained from 1 mol. BAL may therefore be attributed to (*a*) impurity of the sample used for standardization and/or (*b*) other reactions taking place concurrently. Table 9 shows that from 1:2-dimercaptopropionic acid, 0·9 mol. thiocyanate is obtained/mol.; in view of the fact that this compound is a crystalline solid it is possible that it is the purest of the three dithiols examined. However, whether the variation in recovery of thiocyanate between 1:2-dimercaptopropionic acid and BAL is due to a difference in purity, or to the reaction taking a different course, is a very open question.

Application of the method to biological fluids

To determine BAL in blood, use has been made of the stability of the BAL-cyanogen chloride compound in acid solution and the fact that it is recoverable from blood after precipitation of protein. In view of the catalytic action of haemoglobin on the oxidation of BAL, cyanogen chloride has been added in isotonic solution and the protein precipitated by the method of Folin (1930). It should be noted that sodium sulphate must be used for the isotonic solution, and not sodium chloride, which acts as a peptizing agent for tungstic acid so that the centrifugates contain protein and colloidal tungstic acid. The addition of cyanogen chloride to blood produces some cyanide but no thiocyanate (Aldridge & Lovatt Evans, 1946). The cyanide is removed by aeration after protein precipitation. Cyanogen chloride is rapidly taken up by proteins in blood, and an excess must therefore be added to ensure a sufficiency for the BAL-cyanogen chloride reaction to go to completion.

In view of the instability of BAL in blood, indirect methods have been used for the determination of its recovery. Degradation curves have been prepared which have been extrapolated to zero time. (Fig. 1). By this means it has been shown that recoveries of 90–95 % may be expected both from whole blood and plasma. The degradation rate in plasma and serum seems to be higher than that in whole blood. Varying the amount of haemoglobin in the plasma or the addition of citrate and/or cyanide as metallic catalyst poisons had little effect on this rate (Figs. 2, 3). It is possible that this may be due to the incorporation of BAL into an oxidation-reduction system, which retards its oxidation in whole blood, while in plasma no such system is present. Thiocyanate blanks should always be carried out, though these are very low in whole blood because thiocyanate is absorbed by whole blood protein precipitates. Determination of 1:2-dithiols in normal whole blood has so far yielded negative results.

Much difficulty has been encountered in the determination of BAL in urine. The urine must be buffered to about pH 7 for the cyanogen chloride reaction and more bromine water must be added in order to leave an excess. Our examination of the recovery of BAL from various urines has given 100 % recovery only from rather dilute samples from human males; with these samples the 'dithiol blank' was zero. With other samples, in which the 'dithiol blanks' were high (Fig. 4), the recoveries were very

low and the degradation curves were an extraordinary shape. On no occasion have we obtained 100 % recovery from female urine. In many cases we have experienced discolorations of the final colorimetric solution, showing a distinctly brownish shade when compared with those obtained with pure aqueous solutions of BAL.

possible. There are two possible reasons for the low recovery (*a*) the analytical method is not estimating the substance owing to interference from other substances, or (*b*) the substance has been converted to some other derivative which is not determined. If (*b*) is true, the determination of the recovery of consecutive additions of the substance some-

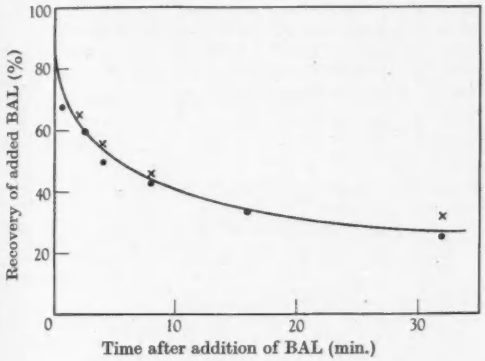


Fig. 1.

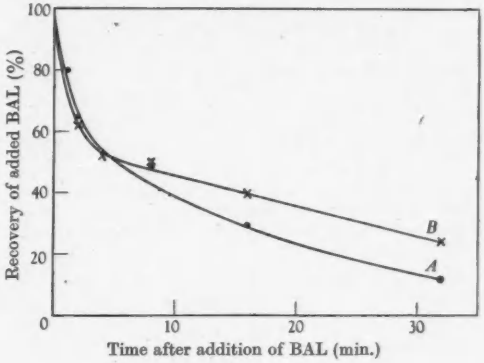


Fig. 2.

Fig. 1. Recovery of added BAL from whole blood. Two determinations plotted.

Fig. 2. Recovery of added BAL from serum. Curve *A*, cat serum (free from haemoglobin); curve *B*, same cat serum with added cyanide.

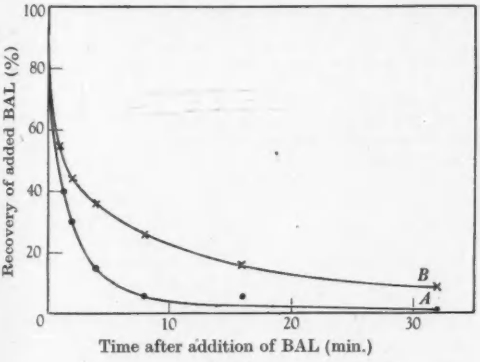


Fig. 3.

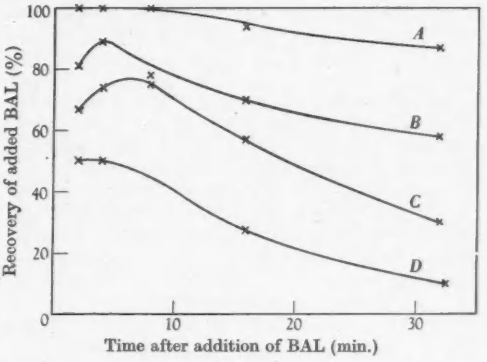


Fig. 4.

Fig. 3. Recovery of added BAL from plasma. Curve *A*, rabbit plasma from defibrinated blood; curve *B*, same rabbit plasma with added citrate.

Fig. 4. Recovery of added BAL from various samples of human urine. Curve *A*, male urine, 'dithiol blank' nil; curve *B*, female urine, 'dithiol blank' 3·6 μg./ml.; curve *C*, male urine, 'dithiol blank' 3·2 μg./ml.; curve *D*, female urine, 'dithiol blank' 11·3 μg./ml. (All 'dithiol blanks' calculated as BAL.)

Upon the recovery of added substances in biological fluids, a few general remarks are necessary. If the recovery of any substance is determined when added to biological media such as blood and urine, theoretical or lower than theoretical results are

times proves the point and the second addition gives a 100 % recovery. However, an experiment of this nature with BAL in urine gave 31 % recovery for the first addition and 46 % for the second.

SUMMARY

1. A new method for the determination of 1:2-dithiols is described, depending on the fact that cyanogen chloride reacts with 1:2-dithiols to give a compound which readily liberates thiocyanate in alkaline solution.

2. 1:3-Dimercaptopropanol and the oxidized form of BAL do not react. Of many thiol compounds, only glutathione, ergothioneine and thiolacetic acid interfere.

3. A method for the determination of BAL in blood is described, but a satisfactory method in the case of urine has not been found. The results obtained are discussed.

4. The mechanism of the reaction is discussed.

I wish to thank the Chief Scientist of the Ministry of Supply for permission to publish this work and Dr L. A. Stocken (Department of Biochemistry, Oxford) for many of the samples of thio compounds. I am also indebted to Mrs L. Currell and Miss A. E. Scott for their painstaking technical assistance; without their keenness this work would scarcely have been possible.

REFERENCES

Aldridge, W. N. (1944). *Analyst*, **69**, 262.

Aldridge, W. N. (1945). *Analyst*, **70**, 474.

Aldridge, W. N. & Evans C. Lovatt (1946). *Quart. J. exp. Physiol.* **33**, 241.

Folin, O. (1930). *J. biol. Chem.* **86**, 173.

Spray, G. H. (1944). Report to Ministry of Supply by Peters, no. 79.*

Spray, G. H., Stocken, L. A. & Thompson, R. H. S. (1945). Report to Ministry of Supply by Peters, no. 85.*

* Information available on application to Ministry of Supply, London.

The Estimation of Urethane (Ethyl Carbamate) in Blood

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In connexion with the recent use of urethane in the treatment of leukaemia (Haddow & Sexton, 1946; Paterson, Ap Thomas, Haddow & Watkinson, 1946), the necessity arose for a method for estimating this substance in blood.

The very simplicity of the chemical structure, $NH_2.COOC_2H_5$, and its close resemblance to urea, indicated that attempts to estimate urethane by reactions based on the amino group were unlikely to be successful. Attention was therefore directed to the characteristic ester grouping. It was found that urethane is quantitatively hydrolyzed to ethanol, ammonia, and carbonate, by boiling with sodium hydroxide solution. The ethanol can be estimated by distillation into acid potassium dichromate solution followed by titration of excess dichromate with sodium thiosulphate after addition of potassium iodide.

METHOD

The estimation involves two stages:

(1) *Determination of volatile reducing substances in blood before alkaline hydrolysis.* Folin-Wu blood filtrate (10 ml.) is measured into a 100 ml. flask containing a few glass beads and connected with a Jackson's condenser which has its delivery tube extended downwards for about 15 cm. The delivery tube dips below the surface of a mixture of potassium dichromate and sulphuric acid (1 ml. of 0·1 N-$K_2Cr_2O_7$ and 5 ml. A.R. conc. H_2SO_4) which is contained in a tube of 20 ml. capacity. The tube containing the mixture is well cooled in ice. With no water cooling on the condenser, about 8 ml. of the flask's contents is distilled over into the oxidizing mixture. A glass stopper is sealed into the tube with a drop of conc. H_2SO_4 and the contents mixed and heated to 80° for 20 min. The tube is then cooled and the contents washed into a flask with about 100 ml. of water, 5 ml. of 5% KI solution are added and the liberated iodine titrated with 0·1 N-sodium thiosulphate.

The above determination usually yields a small blank value.

(2) *Determination of volatile reducing substances in blood after alkaline hydrolysis.* A clean flask is fitted to the condenser, and a mixture of 10 ml. of blood filtrate together with 5 ml. of 10 N-NaOH is refluxed for 15 min. The water supply to the condenser is then stopped and 8–10 ml. of the flask's contents distilled into acid dichromate mixture as in (1). The subsequent procedure is as described in (1) above.

The difference between the dichromate reduced in (1) and in (2) is a measure of the urethane content of the blood. 1 ml. of 0·1 N-dichromate is equivalent to 2·225 mg. of urethane.

RESULTS

As an illustration of the use of the method the results obtained on specimens of blood from two patients with leukaemia who received urethane orally are given.

Case 1. The patient had been receiving 1 g. of urethane daily for 50 days, the last dose being administered at 4.30 p.m. on the 50th day. On the 51st day, a sample of blood was drawn at 1 p.m. At 1.20 p.m. the patient ingested 2 g. of urethane. Blood samples were taken after this dose at 0·5, 2, 5·5 and 22·5 hr. The urethane content of these samples is shown in Table 1.

Table 1. *Blood urethane values of case* 1

Sample	Time (hr.)	Urethane (mg./100 ml.)
1	0	10
2	0·5	38
3	2	36
4	5·5	22
5	22·5	21

The urethane content of the first sample was produced by the preliminary treatment with urethane. Administration of 2 g. raised the level of urethane in the blood by 28 mg./100 ml. in the first 0·5 hr.

Case 2. Samples of blood were obtained before the patient had started urethane treatment. Urethane (3 g.) was then given daily for 14 days and the content of the blood measured at intervals. The results of these estimations are shown in Table 2.

Table 2. *Blood urethane values of case* 2

(Samples 1 and 2 were taken before starting urethane treatment.)

Sample	Day of treatment	Urethane (mg./100 ml.)
1	− 4	4
2	− 2	5
3	1	10
4	3	31
5	7	27
6	14	27

If the pretreatment blank values (samples 1 and 2) are taken as 5 mg./100 ml., then the daily ingestion of 3 g. of urethane maintained in this patient a blood level of about 22 mg./100 ml.

DISCUSSION

Theoretically, the method of estimation described above has the disadvantage that blood may contain compounds other than urethane that, on hydrolysis with alkali, may yield volatile oxidizable substances. In practice, however, it was found that normally this blank value was only about 3–5 mg./100 ml. of blood and did not interfere appreciably with the estimation. Care must be taken, however, that the patient is given no other drugs that may be so hydrolyzed, nor must the blood be preserved with such a substance. Heparin was found quite satisfactory as an anticoagulant for this purpose. It is essential that the blood samples should be as fresh as possible.

Attempts were made to shorten the procedure by carrying out the control and the urethane determinations on the same sample of filtrate. Blood filtrate was distilled down without collecting the distillate in order to remove volatile oxidizable materials. The volume was then restored by addition of water, 10N-NaOH added, and the mixture refluxed for 15 min. before distillation into dichromate. This method proved to be unreliable owing to the volatility of urethane. Up to 50% of the total urethane was lost in the preliminary removal of the volatile substances. In view of this the standard procedure adopted was that described above in which the total urethane and the blank value are determined on separate samples of blood filtrate.

SUMMARY

1. A method is described for the estimation of urethane in the blood of patients undergoing therapy with this drug.

2. The urethane content of the blood can be maintained at a relatively high level for a prolonged period by the daily administration of 1–3 g. of urethane orally to an adult subject.

We are indebted to Prof. A. Haddow and Miss J. M. Watkinson for some of the blood specimens used in this work.

REFERENCES

Haddow, A. & Sexton, W. A. (1946). *Nature, Lond.*, **157**, 500.

Paterson, E., Ap Thomas, I., Haddow, A. & Watkinson, J. M. (1946). *Lancet*, **1**, 677.

Purification of Yeast Hexokinase and its Reaction with $\beta\beta'$-Dichlorodiethyl Sulphide*

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The enzyme theory of vesication, as developed by Peters and Dixon and their respective schools, provides that the primary attack of a vesicant is upon one or more specially sensitive enzymes whose destruction leads to the arrest of some essential metabolic processes of the cell. The vesicant action of compounds such as lewisite and mustard gas ($\beta\beta'$-dichlorodiethyl sulphide, described hereafter as *H*) have thus been studied in relation to their effect upon respiring tissue slices or *brei*, and also upon isolated enzyme systems concerned with intermediary carbohydrate metabolism. In this laboratory, we were more immediately concerned with the role of mustard gas in the inhibition of skin glycolysis, and attention became focused upon hexokinase when there was found to be a correlation between the fall of skin glycolysis, the onset of skin damage, the lowered hexokinase activity of extracts of such skin, and the sensitivity of hexokinase to mustard gas *in vitro* (Dixon, Needham & van Heyningen, 1942; see also review by Dixon & Needham, 1946).

At this stage it was thought desirable to study the action of the vesicant upon the pure enzyme, which could only be obtained in reasonable amount from yeast. The limited objective was to find whether a sensitive enzyme under comparable conditions of treatment reacts more readily than do other proteins whose amino-acid composition is approximately known; the further objective, not yet achieved, was to establish the nature of the sensitive group, assumed to be the active enzyme centre.

Part 1 of the present paper describes the purification, crystallization and properties of yeast hexokinase, and Part 2 describes the action of SH poisons and vesicants. The effect of SH poisons (including lachrymators) is only summarily reported, since the results with the pure enzyme confirm the earlier work of van Heyningen (1942) using crude hexokinase. The inhibition by *H* on the other hand, is described in some detail, and embraces two types of experiment; first, the relation between extent of inhibition and inhibitor concentration under varying conditions, and secondly, the quantitative assay of the number of vesicant groups combined with the enzyme at the point of complete, or almost complete, inactivation. With the small amount of crystalline enzyme at our disposal, this work was only possible by use of mustard gas containing radioactive sulphur (*H**), and by the collaboration of Drs Boursnell, Francis and Wormall, who kindly determined the radioactive S content (*S**) of hexokinase thus inactivated. Under these 'mild' conditions of inhibition, when the enzyme is subjected to a solution of vesicant (as distinct from the more drastic treatment with droplets of liquid vesicant, cf. Herriott, Anson & Northrop, 1946), the uptake of mustard gas by hexokinase was compared with that of other proteins of well defined amino-acid composition and containing a representative number of reactive side-chain groups, viz. ovalbumin and fibrinogen. Moreover, a comparison was made of the amount of vesicant combined with ovalbumin after both mild and drastic treatment, in order to obtain some estimate of the maximal number of vesicant groups which can combine with a protein as compared with the number necessary for enzymic inactivation.

PART 1. PURIFICATION AND CRYSTALLIZATION OF YEAST HEXOKINASE

Yeast hexokinase was partially purified by Meyerhof (1927), van Heyningen (1942) and Dixon & van Heyningen (unpublished), the latter employing adsorption on calcium phosphate. The present authors then continued the research at a stage when American workers had also taken up the problem of vesication. The crystallization of hexokinase was first reported by Cori, Colowick, Berger & Slein (1942), using methods involving ethanol precipitations, and later by Northrop, Kunitz & McDonald (1942) (see also Kunitz & McDonald, 1945–6), using mainly ammonium sulphate fractionation. Our method was developed independently and differs from those of the American workers, but in the last stage of purification we made use of the Kunitz-McDonald newly reported and elegant method for the final crystallization. The activity and crystalline

* This work formed part of the research programme carried out for the Ministry of Supply by an Extra-Mural Research Team under the direction of Dr M. Dixon, and was reported to the Ministry of Supply (Bailey & Webb, 1944*a*).

form of our preparation were similar to those of the crystals which Dr Northrop kindly sent us. It is not clear whether the 'hexagonal plates' obtained by Cori and co-workers represent the same or a different crystalline form, but after crystallization by the Kunitz-McDonald method they are similar to their and our crystals (Berger, Slein, Colowick & Cori, 1945–6).

Activity estimations

The decrease in easily hydrolyzable phosphate during the incubation of the enzyme with adenosinetriphosphate (ATP), glucose and Mg^{++} was determined by the Fiske-Subarrow method, after hydrolyzing the digest with N-HCl for 7 min. at 100°. The method in principle is essentially that given by van Heyningen (1942). The incubation mixture contained 0·5 ml. 0·1 M-$MgCl_2$, 0·6 ml. M-glucose, enzyme diluted to 0·5 ml., and 0·1 M-veronal-acetate buffer pH 7·9 to a total volume of 4·5 ml. Sodium-ATP solution (0·5 ml.) containing *c.* 1·2 mg. 7 min. P was added in the cold, and the mixture placed in the bath at 38°. The amount of enzyme taken was sufficiently small to give an approx. linear breakdown for 7–8 min. Samples of 1 ml. were removed at 1, 3, 5 and 7 min. and pipetted directly into 0·12 ml. 10 N-HCl. After immersion in a boiling water bath for 7 min., each sample was washed into a 50 ml. flask, mixed with Fiske-Subarrow reagents, diluted to volume, and the orthophosphate determined colorimetrically against a standard containing 0·2 mg. P in 50 ml.

One hexokinase unit (HK unit) was defined as the amount of enzyme which transfers 1 mg. 7 min. P from ATP to glucose in 1 hr. under the conditions of the above test, the initial linear portion of the curve 7 min. P against time being used.

Purification of hexokinase

Stage 1. *Autolysis of baker's yeast.* Preliminary experiments showed that autolysis of yeast with toluene and water takes place in two stages. No hexokinase activity is given in the standard test by a suspension of fresh baker's yeast, but yeast treated with toluene for 30 min. at 38° shows a maximal activity soon after mixing with water if the whole suspension is used. At this point, very little of the enzyme is in true solution but is merely accessible to the substrate. On more prolonged incubation of the autolysate, the enzyme gradually passes into solution, and eventually the activity of the supernatant liquid equals that of the whole suspension. Prolonged autolysis at 38°, however, causes considerable destruction of enzyme (up to 50% in 2 days) before extraction is complete (Exps. 1*a*, *b*, Table 1). If, after the preliminary toluene treatment for a few hours at 38°, the autolysis is allowed to proceed at room temperature, destruction losses are smaller, especially in presence of 1% glucose, but the activity of the supernatant liquid usually attained its maximum only after several days. The general effect of glucose, whilst conferring stability, was to retard autolysis (Exps. 2*a*, *b*).

It was then found that addition of SH compounds (cysteine or neutralized Na_2S) had the combined effect of stabilization, accelerating hydrolysis and increasing the final yield of liberated hexokinase (Exps. 3*a*–4*b*). (These results suggest that English baker's yeast is much more

Table 1. *Liberation of 'fixed' hexokinase from yeast under varying autolysis conditions*

	Conditions of autolysis			Hexokinase content (units/ml.)	
Exp.*	Temp. (° C.)	Additions	Duration (hr.)	Whole suspension	Supernatant liquid
1*a*	38	None	18	34	14
			42	14	Nil
1*b*	38	1% glucose	18	21	8
			42	10	Nil
2*a*	20–25	None	18	62	18
			42	40	43
2*b*	20–25	1% glucose	18	45	13
			42	45	23
			66	45	35
			90	43	48
3*a*	20–25	1% glucose	42	38	26
			96	43	31
3*b*	20–25	0·1 M-cysteine	42	56	42
			96	53	53
3*c*	20–25	0·1 M-NaSH	42	42	46
			96	51	42
4*a*	20–25	None	18	51	12
			42	(39)	26
			66	50	34
4*b*	20–25	0·1 M-NaSH	18	49	41
			42	66	58

* Different yeast samples behave differently under standard conditions of autolysis. Since various samples were used, comparison can only be made between results of each individual numbered experiment.

resistant to autolysis than the yeasts used by the American workers. Similar differences between Delft and Manchester yeasts have been noted by Bach, Dixon & Zerfas (1942, 1946) in the preparation of lactic dehydrogenase. Prof. F. Dickens (private communication) has experienced similar difficulties in extracting hexokinase from Distillers Company Ltd. baker's yeast. The earlier work of van Heyningen (1942) suggests that the role of SH compounds in the stabilization of the enzyme may be the prevention of oxidative destruction; the acceleration of autolysis, on the other hand, may be due to their activating effect on proteolytic enzymes which liberate hexokinase from the particulate components of the cell.

The autolysis was carried out in batches as follows: 28 lb. (12·7 kg.) of baker's yeast (Encore) were crumbled, mixed with 760 ml. toluene and maintained at 38° for 3–5 hr. To the liquid mass were added 12 l. of cold tap water, together with 100 g. $Na_2S.9H_2O$ and enough conc. HCl to restore the pH to 6–6·5 (*c.* 50 ml. 10 N-HCl). The mixture was centrifuged after standing for 42 hr. at 20–25°, and the supernatant liquid treated with solid $(NH_4)_2SO_4$ to 75% saturation. The protein was filtered off under suction and stored as a moist filter cake at 0°. Altogether, 185 lb. (84 kg.) of yeast were worked up in this way.

Stage 2. *Dialysis.* The collected filter cakes were dissolved in an equal volume of water. An attempt to fractionate with $(NH_4)_2SO_4$ was abandoned, as the precipitate could not be spun or filtered. The liquid (9·4 l. containing 4,350,000 HK units) was dialyzed against distilled water at 1° in 40 cellophan sacs. After changing the water daily, the $(NH_4)_2SO_4$ concentration fell after 7 days to 0·55 g./l.

Dialysate: 12·8 l.; 3,300,000 HK units; 56 units/mg. N.

Stage 3. *Adsorption.* (Here, as in subsequent adsorptions, small scale experiments were first carried out. A known volume of calcium phosphate gel was added to the enzyme solution and spun down; after determining the enzyme activity of the supernatant liquid, a further portion of gel was added and the whole process repeated until the hexokinase was entirely adsorbed. In the main solution this procedure was shortened by adding just the amount of gel to take down impurities, and after centrifuging, a subsequent portion to adsorb most of the enzyme.) The dialysate (pH 6) was stirred with 5 l. of calcium phosphate gel (4·4% dry wt.), which was spun off and discarded. The enzyme was adsorbed on a further 5 l. of gel, and after centrifuging, the precipitate was eluted six times with Na_2SO_4 solution (100 g. anhydrous salt/l.); 30 g. glucose were added to the eluates to stabilize the enzyme.

Eluates: 7·13 l.; 1,750,000 HK units; 113 units/mg. N.

Stage 4. *Ammonium sulphate fractionation.* Solid $(NH_4)_2SO_4$ was added to give 55% saturation. (Here again, the conditions for fractional salting out were determined in small-scale experiments.) The precipitate was filtered off under gravity and rejected. The salt concentration was then increased to 75% saturation, the precipitate filtered off, dissolved in 1% glucose solution and dialyzed against several changes of 1% glucose at 0° for 3 days.

Dialysate: 885 ml.; 860,000 HK units; 264 units/mg. N.

Stage 5. *Adsorption.* The solution was treated with 88 ml. calcium phosphate gel, spun, and the precipitate rejected. Another 550 ml. of gel were added, spun off, and the precipitate eluted with Na_2SO_4-glucose solution (100 g. anhydrous sodium sulphate, 10 g. glucose/l.).

Eluates: 925 ml.; 670,000 HK units; 550 units/mg. N.

Stage 6. Attempts to increase the activity by fractional salting-out, or to crystallize the enzyme were unsuccessful. Eventually all fractions were combined, salted out with $(NH_4)_2SO_4$ (85% saturation), filtered under suction, taken up in a small volume of water and dialyzed against 0·1 M-acetate buffer at pH 5·9 and containing 1% glucose. About 210,000 HK units at 300 units/mg. N resulted from these unsuccessful trials. In future preparations, these losses would be avoided by passing on to the next step, thereby increasing the eventual yield threefold.

Stage 7. *Ethanol precipitation.* Absolute ethanol at −10° was added to the cooled solution (98 ml.) to give a final concentration of 34% (v/v). The precipitate was centrifuged down at 0° and discarded. The ethanol content of the supernatant liquid was raised to 53% (v/v), the precipitate centrifuged down, dispersed immediately in 1% glucose, and insoluble material separated by filtration through paper pulp. All operations were carried out at or near 0°.

Solution: 172 ml.; 115,000 HK units; 400 units/mg. N.

Stage 8. *Crystallization.* Although at stage 7 the preparation was less pure than at stage 5, it yielded readily to the crystallization procedure used by the Northrop school. It seems very probable that the ethanol solutions removed lipid material which prevents crystallization. The solution from stage 7 was salted out in 85% saturated $(NH_4)_2SO_4$ solution, and the protein obtained as a filter cake. It was dissolved in a small quantity of 0·1 M-phosphate buffer pH 7, saturated ammonium sulphate was added to a faint turbidity, and the liquid allowed to drain through a filter at 0°. After a week, crystals (A) appeared in the filtrate. The residue on the paper was again dissolved and treated as above, and yielded a second crop (B). The crystals were washed in 75% saturated $(NH_4)_2SO_4$ solution, and dissolved for activity tests in 3% Na_2SO_4 containing 1% glucose.

Crystals A: 4 mg. protein N; 6200 HK units; 1530 units/mg. N.

Crystals B: 13 mg. protein N; 15,600 HK units; 1200 units/mg. N.

From the mother liquors, further small crops were obtained which eventually were combined with the main crops and recrystallized.

The final product, reproduced in Pl. 1, contained 13 mg. protein N (i.e. 83 mg. protein) and 40,000 HK units at 2500–3000 units/mg. N. The crystals were stored moist in saturated ammonium sulphate solution at 0°.

Comparison of the activity of the crystals with the preparations of Cori and of Northrop. Northrop *et al.* (1942) reported an activity of 1440 Cori units/mg. protein for five times recrystallized hexokinase, i.e. 9230 Cori units/mg. protein N. Assuming a temperature coefficient for the enzyme reaction of $Q_{10}=2$, calculation shows that 1 Cori unit (defined as the amount of enzyme catalyzing the formation of acid equivalent to 1 μl. CO_2/min. at 25°) is equal to 0·205 of our HK unit (defined as the amount causing transfer of 1 mg. P/hr. at 38°). Using this conversion factor, Northrop's reported activity is equal to 1890 HK units/mg. N.

Cori, Colowick, Berger & Slein (1943), however, later reported an activity of 2100 Cori units/mg. protein for a sample of hexokinase obtained from

Northrop. Converted to our units, this corresponds to an activity of 2750 HK units/mg. N. In agreement with this value, a sample of Northrop's crystalline hexokinase, kindly made available to us by Prof. Peters, gave an activity of 2850 HK units/mg. protein N under our test conditions. These figures are similar to those for our own preparation.

Summarization of purification data. The quantitative data on the purification process are brought together in Table 2. The early steps of alternate salting out and adsorption give a much more rapid purification than the repeated salt fractionation adopted by Northrop and co-workers. In the one case, the activity is roughly doubled at each stage, in the other, a 25 % increase in purity was achieved.

The pure, recrystallized preparation represents a recovery of *c.* 1·2 % enzyme and a 53-fold purification on the dialysate of stage 2. With our present procedure, the yield could possibly be increased to 3·6 % by passing from stage 5 to 7.

above pH 7: 5 min. at 38° and pH 7·9 sufficed to inactivate completely, whilst at the same pH and 20°, 90 % of the activity was lost in 2·5 hr. No evidence of protection from spontaneous inactivation was observed with glycine or with cyanide, although the latter salt was stated by van Heyningen (1942) to act as stabilizer.

The enzyme was inactive in absence of Mg ions, and these could not be replaced by Ca^{++}. The activity in presence of 5×10^{-3}M-Mg^{++} was not affected by the addition of an equal concentration of Ca^{++}; the Ca : Mg antagonism which is so pronounced in the case of the Mg-activated yeast pyrophosphatase (Bailey & Webb, 1944*b*) is thus absent in the case of hexokinase. It would be of interest to discover whether such antagonism as is found in yeast pyrophosphatase and myosin adenosinetriphosphatase is general for those enzymes producing free phosphate, but absent in the phosphate-transferring enzymes (phosphokinases). The proteins oval-

Table 2. *Purification of yeast hexokinase*

Stage	Volume (l.)	Total activity* (hexokinase units)	Purity (units/mg. N)	Total N* of fraction (g.)
2. Filter cake from 84 kg. yeast	10·9	$4·7 \times 10^6$	—	—
Same after dialysis	12·8	$3·3 \times 10^6$	56	59·0
3. Adsorption and elution	7·13	$1·75 \times 10^6$	113	15·5
4. $(NH_4)_2SO_4$ fractionation	0·885	860,000	264	3·25
5. Adsorption and elution	0·925	670,000	550	1·22
6. Unsuccessful fractionation; fractions combined	0·215	210,000	300	0·70
7. Ethanol precipitation	0·172	115,000	400	0·30
8. Crystals A	—	6,200	1530	0·004
Crystals B	—	15,600	1200	0·013
Recrystallized (A, B and other crops)	—	40,000	2500–3000	0·013

* Activity and N (micro-Kjeldahl) determined on dialyzed material; corrections for traces of NH_3-N applied after distilling undigested sample at pH 9·5 in the micro-Kjeldahl apparatus.

Properties of hexokinase

An extensive examination of the properties of hexokinase was not undertaken, since the material was required for combination with $\beta\beta'$-dichlorodiethyl sulphide containing radioactive S. Moreover, the general properties of the enzyme (metal activation, stability, pH optimum, substitution of other sugar substrates for glucose) had already been determined for a partially purified preparation by van Heyningen (1942). Similar investigations have also been made by Berger *et al.* (1945–6) and by Kunitz & McDonald (1945–6) on the crystalline enzyme.

Our own preparation dissolved in water to give a colourless solution. Except in high salt concentration or in presence of glucose the enzyme was rapidly inactivated (without visible precipitation) bumin and insulin, found by Cori *et al.* (1943) to produce activation of the enzyme, were quite ineffective with our preparation. As compared with the theoretical value of 50 %, about 43 % of the easily hydrolyzable P of ATP was transferred to glucose in presence of the enzyme in accordance with the generally accepted view that hexokinase catalyzes the reaction:

Adenosinetriphosphate + glucose
$\rightleftharpoons$ Adenosinediphosphate + glucose-6-phosphate

Physical constants for the enzyme have been obtained by Kunitz & McDonald (1945–6). The protein was found to be electrophoretically pure, and to give a solubility independent of the amount of solid phase. Cataphoretic studies gave an isoelectric point between pH 4·5–4·8, and sedimentation-diffusion a molecular weight of 96,600.

PART 2. INHIBITION OF HEXOKINASE BY SH POISONS AND BY $\beta\beta'$-DICHLORODIETHYL SULPHIDE (*H*)

SH *poisons including lachrymators*

All SH poisons, including lachrymators and the vesicant lewisite, were most powerful inhibitors, and much more effective than iodoacetate. Of inhibitors unrelated to vesicants and lachrymators, 0·04 M-NaF gave a 46 % inhibition under the standard test conditions.

In Table 3 are listed the inhibitions obtained with varying concentrations of SH poisons. Except where otherwise stated, the poisons were incubated with enzyme in veronal-acetate buffer pH 7·9 containing $1{\cdot}1 \times 10^{-3}$ M-$MgCl_2$ and M/73 glucose. Additional glucose (to raise the concentration to that used in the standard activity assay) was added with the ATP after this preliminary incubation. The marked inhibition which occurs at very low concentration in the case of chloroacetophenone, bromobenzyl cyanide, ethyl iodoacetate and lewisite, known from the work of Mackworth (1941, 1947) to be SH-combining reagents, is in general agreement with the results of van Heyningen (1942) carried out with an impure preparation, and support her conclusion that the presence of SH groups in the hexokinase molecule is essential for its activity.

Table 3. *Effect of* SH *poisons on hexokinase*

Inhibitor	Concentration of inhibitor	Conditions of preliminary incubation: Time (min.)	Conditions of preliminary incubation: Temp. (° C.)	Inhibition (%)
Chloroacetophenone	M/880	15	38	33
	M/880	15	38	50
	M/880	5	16	Nil
Bromobenzyl cyanide	M/800	5	38	100
Ethyl iodoacetate	M/800	5	38	49
Na iodoacetate	M/63	5	38	100
	M/200	5	38	20
Lewisite (in M/8 glucose)	M/1000	15	38	100
	M/2000	15	38	70
	M/2500	15	38	43
	M/4000	15	38	20

The inactivation of crystalline hexokinase by radioactive H (H)*

It was our aim to treat the enzyme with *H* under the mildest conditions consistent with a high degree of inactivation, so as to reduce to a minimum any general attack which might conceivably accompany the inactivation process, although playing no essential part in its causation. Preliminary experiments were carried out with non-radioactive *H* in order to determine these conditions. The inherent instability of the enzyme, however, creates special difficulties. van Heyningen (1942) reported an 80 % inactivation by $2{\cdot}5 \times 10^{-3}$ M-*H* (initial concentration) in 0·014 M-veronal-acetate buffer at pH 7·8 and 38° in presence of 0·007 M-glucose. Under these conditions, a considerable amount of spontaneous inactivation occurred in the control without *H*, and although this was allowed for in the calculation of percentage inhibition, it is essential for our present purpose that any inactivation other than that due to *H* should be eliminated as far as possible. Without this provision, any comparison between extent of inactivation and the number of *H* groups combining is meaningless.

Unfortunately, the conditions for greatest sensitivity to *H* are also those of least stability. Enzymes in general seem to become more sensitive to *H* as the pH is raised (see Dixon (1943) and Table 4), but with hexokinase, pH values above 7 cannot be employed at temperatures higher than 20° because of the spontaneous inactivation which then occurs. The enzyme is more stable at lower temperatures, but these necessitate a long incubation with *H* to allow of complete reaction. Again, the presence of glucose, whilst effecting stabilization, reduces the sensitivity of the enzyme to *H* (Dixon, 1943). With reasonably small amounts of glucose (*c.* 0·01 M) the inactivation by *H* could be measured without serious spontaneous inactivation, either by incubation at 38° in buffer at pH 5·5, or by treatment at room temperature in buffer at pH 7·4–7·9. A few results with crystalline hexokinase are shown in Table 4; similar results were obtained with a crude yeast autolysate and with a partially purified preparation. In each case, a solution of *H* in ethylene glycol monoethyl ether was added to a dilution of enzyme sufficient for one test, to give a solution of concentration $2{\cdot}5 \times 10^{-3}$ M. The activities after incubation were measured at pH 7·5 or 7·9 and compared with a control treated similarly with *H*-solvent in absence of *H*.

Table 4. *Inhibition of crystalline hexokinase by H*

pH	Temp. (° C.)	Time of incubation with $2{\cdot}5 \times 10^{-3}$ M-*H* (min.)	Composition of incubation mixture: Buffer	Glucose (M)	Mg^{++} (M)	Enzyme N/ml. (μg.)	Inhibition (%)
7·5	16	135	0·08 M-veronal-acetate	0·011	0	2·4	25
7·9	20	15	0·09 M-veronal-acetate	0·0001	0·013	1·2	44
5·5	38	15	0·1 M-acetate	0·0056	0	2·8	13
5·5	38	15	0·1 M-acetate	0	0	1·4	10

From Table 4 it will be seen that hexokinase is comparatively insensitive to *H* at pH 5·5, even in the absence of glucose. At the higher pH values and lower temperature, the inactivation is much more marked, and the protection by glucose is in evidence. It was clear from these results that it was not possible both to obtain full sensitivity to *H* and freedom from spontaneous inactivation, and that a compromise must be sought. It was finally decided to sacrifice sensitivity to some extent in order to reduce the spontaneous inactivation to a reasonably small value, and to attain the necessary degree of inhibition by treating the enzyme several times in succession with small amounts of *H*.

Treatment of hexokinase with radioactive H. The compromise conditions for obtaining a reasonable sensitivity to *H* and low spontaneous inactivation were realized by treating with *H* at room temperature at pH 7·4 in presence of M/180 glucose. Additions of radioactive *H* in an organic solvent were made at intervals of about 45 min. until a sufficient degree of inactivation had been obtained, each addition in the concentration of $2{\cdot}5 \times 10^{-3}$ M. A control without *H* gave a measure of the spontaneous inactivation. With the amount of crystalline enzyme available (80 mg.) only two experiments could be carried out. These are described below.

Exp. 1. Crystalline hexokinase containing 5·1 mg. protein N was dissolved in 5 ml. 0·1 M-phosphate buffer pH 7·4 containing M/180 glucose. For activity measurements, 0·01 ml. samples were pipetted into 1 ml. of ice-cold water and 0·1–0·3 ml. of this solution was used for each test. At various intervals, shown in Table 5, 0·05 ml. of 0·25 M-*H* in ethylene glycol monoethyl ether was added, until the activity fell to a fairly low value. No strictly parallel control to determine the extent of spontaneous inactivation was made in this experiment, but the first diluted sample, taken before *H* addition, was incubated under the conditions of the main experiment and used as a diluted control; this procedure is open to criticism since dilution may have the effect of decreasing stability, the control thus giving too high a value for spontaneous inactivation. In Exp. 2, therefore, this defect was remedied by incubating a control under the same conditions as those of the experimental solution.

Exp. 2. The enzyme (6·8 mg. protein N) was dissolved in 5·5 ml. 0·1 M-phosphate buffer pH 7·4 containing M/180 glucose. A 5 ml. sample was used for *H* treatment and 0·5 ml. incubated similarly as a control without *H*, the same concentration of *H* solvent (in this case ethanol) being added. The results are shown in Table 5.

In both experiments, the *H*-treated solutions remained clear and gave no visible evidence of denaturation.

Table 5. *Treatment of crystalline hexokinase with radioactive H*

(0·05 ml. 0·25 M-*H** added to final concentration of $2{\cdot}5 \times 10^{-3}$ M at times marked →.)

Exp. no.	Time of activity determination (hr.)	Activity of *H**-treated enzyme as % of original (HK units/ml.)	Activity of control as % of original (HK units/ml.)
1	→ 0	100	100
	→ 0·75	95	—
	→ 1·75	—	—
	→ 2·5	40	—
	3·25	23	77*
2	→ 0	100	100
	→ 0·5	—	—
	→ 1·25	—	—
	→ 1·5	—	—
	2·0	31	97
	→ 3·0	—	—
	3·75	29	91

* 'Diluted' control, see text.

Determination of combined H. The *H*-treated hexokinase from each experiment was precipitated with an equal volume of ethanol and allowed to settle overnight. It was washed thrice with 50% (v/v) ethanol and twice with absolute ethanol. After Soxhlet extraction with acetone and subsequently with ether to remove any *H** and thiodiglycol containing *S**, it was dried over $CaCl_2$. Protein N was determined by the micro-Kjeldahl method on a sample of the dried powder after removing non-protein N with 5% trichloroacetic acid, and the remainder was used for *S** determinations. These latter were carried out by Drs Boursnell and Francis (cf. Boursnell, Francis & Wormall, 1946) using the Geiger counter method as adapted by Banks (1946). From the N and *S** determinations the number of *H* molecules combining with each molecule of enzyme was calculated, taking the N content of ash-free and moisture-free hexokinase as 15·6%, and the molecular weight as 97,000 (Kunitz & McDonald, 1945–6). The results are shown in Table 6. For reasons

given above, the control in Exp. 2 has been used in the calculations for Exp. 1, rather than the 'diluted' control. Since both experiments were carried out under very comparable conditions, the rates of spontaneous inactivation could have differed only slightly. The number of *H* groups/molecule of enzyme inactivated by *H* is calculated on the assumption that the degree of inactivation is proportional to the *H* groups combined. In both experiments this number corresponds to 6–7 groups/molecule enzyme, or, assuming the average amino-acid residue weight to be comparable to that of a polar

Table 6. *Estimation of H* groups combined with hexokinase*

	Exp. 1	Exp. 2
Protein-N content of powder (%)	10·62	11·5
Wt. taken for *S** determination (mg.)	35·5	(*a*) 20·75 (*b*) 17·45
*S** found (as mg. *H**)	0·169	(*a*) 0·105 (*b*) 0·094
Molecules *H**/molecule hexokinase	4·3	(*a*) 4·2 (*b*) 4·4 Mean 4·3
Total enzyme inactivation (%)	77	71
Inactivation in control (%)	(9)	9
Inactivation due to *H* (%)	68	62
Estimated no. *H* groups/molecule hexokinase for 100% inactivation by *H*	6·3	7·0

protein such as fibrinogen, i.e. 114, to the combination of less than 1 *H* group in 100 amino-acid residues. By comparison, the extent of substitution when the enzyme is stirred with liquid *H* (at pH 5·5–6·0) can be gauged from the work of Herriott *et al.* (1946) who found that 30–35 *H* groups combined with the enzyme. This latter figure suggests a general attack upon the enzyme, whilst our own much lower figure is scarcely more than might be expected from a specific combination of *H* with the active enzyme centres.

The reaction of other soluble proteins with H

An enzyme may be particularly sensitive to *H* either because (*a*) it contains specially reactive groups, and is capable of substitution under conditions which are without effect on insensitive enzymes and presumably on proteins in general, or because (*b*) although the reactivity may be similar to that of other proteins, the reacting groups are specially connected with enzyme activity. To distinguish between these possibilities in the case of hexokinase it seemed desirable to compare the behaviour of other proteins, using the same mild conditions of *H* treatment. The proteins selected were thrice recrystallized ovalbumin, and six times precipitated ox fibrinogen.

Conditions of H treatment. The proteins were dissolved in phosphate buffer at pH 7·5 to give a concentration of 7–8 mg./ml. A solution of *H* in ethanol (0·25M) was added in five successive portions at intervals of 0·75 hr., each portion being 1/100 of the volume of the original solution. The temperature was maintained at 20°. Under these conditions, the *H* was in clear solution, and throughout the reaction the sols remained free of precipitated protein. After treatment the sols were dialyzed, precipitated with an equal volume of acetone after adjusting to pH 5·5, washed four times with acetone, four times with ethanol, and finally with ether. Samples of untreated protein were similarly precipitated and washed, and finally dried over $CaCl_2$ for N and S determinations.

For comparison, ovalbumin was also treated with liquid *H*. The protein (4·5 g.) in 80 ml. water was stirred with 1 ml. of liquid *H*, dispersed in fine droplets, for 10 hr. at 30°; solid $NaHCO_3$ was added at intervals to maintain the pH at 7. The *H*-treated sol was then decanted from residual *H*, dialyzed against water, adjusted to pH 5·5, precipitated with 10 vol. acetone, and Soxhlet-extracted for 6 hr. each with acetone, ethanol and ether.

Sulphur determinations. The HNO_3-$KClO_3$-$Cu(NO_3)_2$ oxidation generally employed for the determination of S in *H*-treated proteins (e.g. Boursnell *et al.* 1946) is lengthy and sometimes violent. The alternative method of alkali fusion gives a very complete oxidation of the S-amino-acids of proteins but with *H*-treated products, it is open to the objection that combined *H* groups may be split off and lost in the form of volatile sulphides or their oxidation products. This difficulty has been overcome by mixing the finely-ground protein (0·3 g.) with a large excess of fusion mixture (8 g. of 1 : 1 Na_2CO_3 and Na_2O_2) and heating in a nickel crucible on a hot-plate at 140–160°. After 0·5–1 hr. the mixture gently fuses, each protein particle burning at red heat in the oxidizing medium. The melt is then heated to redness for 3–4 min., cooled, and sulphate estimated gravimetrically using glass-distilled water. The reagent blank amounted to 1 mg. $BaSO_4$. The S content of crystalline *H* sulphoxide determined by this method was 18·4% (theory, 18·3%).

The combination of H with ovalbumin and fibrinogen. The number of *H* groups in combination with 10^5 g. protein (chosen as a basis for comparison with hexokinase, which has a molecular weight of nearly 10^5) was calculated from the expression

$$\frac{1000\,N^0}{32}\left(\frac{S^H}{N^H}-\frac{S'}{N'}\right),$$

where S' and N' are the S and N contents of the untreated protein (not necessarily dry); S^H and N^H are the values for the *H*-treated sample, and N^0 the true N percentage of the original ash-free protein dried at 105°. The results are summarized in Table 7. Despite the fact that the analytical methods used cannot determine the extent of substitution with any great precision when only small differences of S content are measured, it is nevertheless clear that the number of *H* groups combining under the 'mild' treatment are comparable with those in hexokinase; such differences as exist can largely be explained on

Crystalline yeast hexokinase (× 135).

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Table 7. *Treatment of ovalbumin and fibrinogen with H*

Protein	Conditions of treatment	Untreated (dried at 105°) N (%)	Untreated (dried at 105°) S (%)	*H*-treated (air-dried) N (%)	*H*-treated (air-dried) S (%)	Mol. $H/10^5$ g. protein
Fibrinogen*	0·025 M-$NaHCO_3$, 0·06 M-phosphate, 0·15 M-NaCl (*H* in solution)	16·7	1·16	(*a*) 15·85	1·32	7·2
				(*b*) 15·9	1·34	7·8
					1·28	5·8
					Mean	7·0
Ovalbumin	0·1 M-phosphate (*H* in solution)	15·5	1·56	14·0	1·55	4·9
					1·59	6·3
					Mean	5·6
Ovalbumin†	$NaHCO_3$ (liquid *H*)	15·5	1·56	12·95	2·19	33
					2·25	35
					Mean	34
Hexokinase (see Table 6)	(*H* in solution)	—	—	—	—	4·3

(*a*) and (*b*) represent separate experiments, not duplicate analyses.

* After *H*-treatment, the fibrinogen sol in presence of purified thrombin still clots; the clotting time is only slightly delayed, but the clot is at first of loose texture and opalescent, and becomes more rigid on standing.

† *H*-treated ovalbumin after 24 hr. at pH 11 and room temperature contains 14·8% N and 1·95% S (air-dried), corresponding to 15 mol. $H/10^5$ g. protein.

the basis of differences in buffer competition. From these experiments, therefore, it appears that the sensitivity of hexokinase to *H* is not due to any special reactivity of the molecule as a whole, but rather to the dependence of enzyme activity upon groups which are relatively easily substituted by *H*.

As against the six *H* groups combined with 10^5 g. ovalbumin after mild treatment, some 34 are taken up when treated with liquid *H*. These figures run parallel with those already discussed for hexokinase under the two comparable sets of conditions. When allowed to stand at room temperature at pH 11 for 24 hr., 19 of the 34 groups were found to be alkali-labile, indicating that 56% of the groups substituted are probably in ester linkage with carboxyl groups. The residual (alkali-stable) groups may involve amino groups, though according to Herriott *et al.* (1946) these are not substituted in the case of ovalbumin. None of the groups was hydrolyzed by a partially purified liver esterase preparation, although this enzyme is relatively non-specific, hydrolyzing almost all esters.

It may be noted that the extent of *H* substitution in the case of ovalbumin treated with liquid *H* corresponds to one *H* group/26 amino-acid residues. It is remarkable, therefore, that the protein evinces no sign of denaturation and remains soluble both at pH 7 and 5, but, like the native form, is readily heat-coagulated at the latter pH. This is in contrast to the behaviour of nucleoproteins which become insoluble after reaction with *H* (Berenblum, 1940). As recent work (Auerbach & Robson, 1946) suggests that nuclear changes are manifest in *H*-damaged tissue, this difference of behaviour may be of great importance to a complete understanding of the toxicology of mustard gas.

SUMMARY

1. The purification and crystallization of hexokinase from baker's yeast are described. The method was developed independently of those of the Cori and Northrop schools, but the procedure of the latter for the final step of crystallization has been followed. The crystals are of the same form and activity as those of Northrop.

2. The addition of cysteine or NaSH to autolyzed baker's yeast accelerates the liberation of hexokinase from the particulate components of the cell and retards inactivation.

3. As found by van Heyningen (1942) for impure hexokinase, the pure enzyme is stabilized by glucose and is activated by Mg ions. This activation is not antagonized by Ca ions.

4. The enzyme is inhibited by SH poisons (including lachrymators), suggesting that hexokinase is an SH enzyme. These results confirm those of van Heyningen (1942) using impure hexokinase.

5. Since vesicant action is accompanied by *in vivo* inactivation of hexokinase (Dixon *et al.* 1942), the uptake of vesicant by yeast hexokinase has been studied, using $\beta\beta'$-dichlorodiethyl sulphide containing radioactive S. By comparison with other proteins treated with vesicant under similar conditions, it is concluded that the sensitivity of hexokinase to mustard gas is not due to any special reactivity, but to the dependence of enzyme activity on groups which are relatively easily substituted.

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REFERENCES

Auerbach, C. & Robson, J. M. (1946). *Nature, Lond.*, **158**, 878.
Bach, S. J., Dixon, M. & Zerfas, L. G. (1942). *Nature, Lond.*, **149**, 48.
Bach, S. J., Dixon, M. & Zerfas, L. G. (1946). *Biochem. J.* **40**, 229.
Bailey, K. & Webb, E. C. (1944*a*). Report to Ministry of Supply by Dixon, no. 30.*
Bailey, K. & Webb, E. C. (1944*b*). *Biochem. J.* **38**, 394.
Banks, T. E. (1946). *Brit. J. Radiol.* **19**, 333.
Berenblum, I. (1940). Report to Ministry of Supply by Peters, no. 16.*
Berger, L., Slein, M., Colowick, S. P. & Cori, C. F. (1945–6). *J. gen. Physiol.* **29**, 379.
Boursnell, J. C., Francis, G. E. & Wormall, A. (1946). *Biochem. J.* **40**, 743.
Cori, C. F., Colowick, S. P., Berger, L. & Slein, M. (1942). By communication.*
Cori, C. F., Colowick, S. P., Berger, L. & Slein, M. (1943). By communication.*
Dixon, M. (1943). Report to Ministry of Supply by Dixon, no. 19.*
Dixon, M. & Needham, D. M. (1946). *Nature, Lond.*, **158**, 432.
Dixon, M., Needham, D. M. & van Heyningen, R. (1942). Report to Ministry of Supply by Dixon, no. 11.*
Herriott, R. M., Anson, M. L. & Northrop, J. H. (1946). *J. gen. Physiol.* **30**, 185.
Kunitz, M. & McDonald, M. R. (1945–6). *J. gen. Physiol.* **29**, 393.
Mackworth, J. F. (1941). Report to Ministry of Supply, by Dixon, no. 4.*
Mackworth, J. F. (1948). *Biochem. J.* **42**, 82.
Meyerhof, O. (1927). *Biochem. Z.* **183**, 176.
Northrop, J. H., Kunitz, M. & McDonald, M. R. (1942). By communication.*
van Heyningen, R. (1942). Report to Ministry of Supply by Dixon, no. 10.*

* Information available on application to Ministry of Supply, London.

The Metabolism of the Lungs from Normal Mice and from Mice Exposed to Phosgene

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The respiration and glycolysis of the lungs of normal mice, and of mice previously exposed to phosgene, have been measured in order to determine if the oedema following exposure is preceded by inhibition of the carbohydrate metabolism. This was done in the hope that knowledge of the nature of the changes might lead to a rational therapy of phosgene poisoning.

EXPERIMENTAL

The measurements were made with the Warburg apparatus at 38°. The lungs were suspended in Ringer-phosphate in the presence of O_2 for respiration measurements and in Ringer-bicarbonate in 95% N_2/5% CO_2 for measurement of the glycolysis. To minimize injury to the lung, the whole lobes from mice of about 20 g. wt. were used for the measurements. Lungs of larger animals would have necessitated the use of minced or sliced tissue. Manometric readings were taken at intervals over a 2 hr. period. The tissue in each experiment was weighed (*a*) before the manometric measurements were made, (*b*) in the wet state at the end of the manometric measurements and (*c*) after drying to constant wt. at 100°. The metabolic quotients (Q) are expressed in μl./mg. dry wt./hr.

RESULTS

Comparison of metabolism of whole lobes and cut lung tissue from normal mice

The lungs of freshly killed normal mice were removed and washed with Ringer solution. The lobes of one lung were weighed, and placed whole in a Warburg vessel, while the lobes of the other lung were minced into fine pieces with scissors before being placed in a second vessel. Some values for metabolism of whole and minced tissue are given in Table 1. The mean values for Q_{O_2} with glucose are 7·3 for whole tissue, 7·4 for minced tissue (eight determinations of each type). Similar experiments have shown that the respiration in the absence of glucose, and the glycolysis in the presence of glucose, are not seriously changed by mincing of the tissue. These results indicate that measurements made on whole lobes of small mice are not affected by the rates of diffusion of O_2 or CO_2 in the tissue and give a true picture of the activity of the enzymes within the tissue. The

slight increase, which is found in the minced tissue, is probably due to the loss of material in the Ringer solution so that the dry weights of tissue are really somewhat low. The values for respiration are similar to those obtained for rat lung by Laser (1932), but the values for glycolysis are higher than those found with rat lung.

Effect of exposure to phosgene

Mice were exposed to air containing phosgene in a chamber as described by Boyland, McDonald & Rumens (1946), the concentrations being checked analytically. The results obtained on the lungs of such exposed mice are set out in Table 2. It is clear

Table 1. *The effect of mincing on the metabolism of normal mouse lung*

Nature of tissue	Wt. of tissue (mg.)		Increase in wet wt. during exp. (%)	Metabolism		
	Before exp. (wet)	Dry		Respiration with glucose Q_{O_2}	Respiration without glucose Q_{O_2}	Glycolysis of glucose $Q_G^{N_2}$
Whole lobes	70	14	52	6·4	—	—
Minced tissue	79	14	133	7·8	—	—
Whole lobes	95	16·2	10	6·9	—	—
Minced tissue	119	18·6	2	6·5	—	—
Whole lobes	65	13·2	48	7·5	—	—
Minced tissue	71	13	12	7·7	—	—
Whole lobes	68	10·6	43	—	7·5	—
Minced tissue	100	17	21	—	8·3	—
Whole lobes	84	17	50	—	6·1	—
Minced tissue	88	15	Nil	—	7·1	—
Whole lobes	74	15	19	—	—	3·0
Minced tissue	69	13	17	—	—	3·8
Whole lobes	85	16	47	—	—	4·0
Minced tissue	120	20	15	—	—	5·4
Whole lobes	82	14	26	—	—	3·5
Minced tissue	89	17	26	—	—	3·6

Measurements of normal lung tissue showed that the respiration is not increased by addition of glucose to the medium. Mean values obtained for Q_{O_2} with glucose were 7·3 (individual values 6·3, 6·3, 7·8, 7·9 and 8·6), and Q_{O_2} without glucose, 7·6 (individual values 6·3, 6·4, 6·5, 6·7, 7·0, 7·2, 7·3, 7·5, 8·0 and 9·1). Similar figures were obtained for the respiration in the presence of DL-alanine, viz. Q_{O_2} without substrate 5·8–7·0, and Q_{O_2} with 0·025M-alanine 6·4–6·8. These results indicate that, if the utilization of glucose or alanine were specifically inhibited by phosgene, such inhibition might not reduce the respiration. The glycolysis values ($Q_G^{N_2}$) found were 2·7, 2·8, 2·9, 2·9, 3·1, 3·1, 3·4 and 3·4, having a mean of 3·0.

In all cases in which measurements were made with lungs from normal mice, the tissue increased in weight over the experimental period. The increase in weight varied from 0 to 133% of the original weight; it is due in part to replacement of air in the lung by Ringer solution, and also to swelling of the tissue.

that there is no significant change in the respiration or glycolysis of the lung tissue following exposure to lethal doses of phosgene.

Most of the lungs of mice exposed to phosgene increased in weight during the measurements, often to the same extent as did normal lungs. In one group of mice which were exposed to a high dosage ($Ct = 4080$ mg. min./m.^{3}, where C = concentration of phosgene in mg./m.^{3} and t = time in min.), 19 hr. before examination, the wet weights of the lungs decreased during the experiment. This was presumably due to oedema fluid, and possibly other material being washed away with Ringer solution while the manometric measurements were being made. It is possible that a reversal of tissue swelling occurred in the Ringer solution.

The change in weight of the lung following exposure could be seen by comparing the weight of the wet lung with the total body weight of the mice. Some estimations of nucleic acid phosphorus were carried out by the method of Berenblum, Chain & Heatley (1939). The data (Table 3) show how the

Table 2. *Metabolism of lungs from mice exposed to phosgene*

Details of exposure (dosages (Ct) in mg. min./m.^{3})	Wt. of tissue (mg.) Before exp. (wet)	Wt. of tissue (mg.) Dry	Change in wet wt. during exp. (%)	Q_{O_2} with glucose	Q_{O_2} without glucose	Glycolysis of glucose $Q_G^{N_2}$
$Ct=2100$; $t=8$ min. (less than 1 hr. before measurement)	70	12·8	—	7·1	—	—
	103	20·2	—	7·7	—	—
	61	8·8	—	9·2	—	—
	124	23·0	+24	6·0	—	—
	92	17·0	—	—	6·4	—
	44	8·2	—	—	6·4	—
	122	23·6	+32	—	6·2	—
	122	22·0	+47	—	6·2	—
	150	29·6	—	—	—	2·5
	195	39·6	—	—	—	2·5
	161	32·0	+49	—	—	3·0
	165	31·4	+36	—	—	2·6
$Ct=5300$; $t=8$ min. (less than 1 hr. before measurement)	108	16·6	+15	7·1	—	—
	116	17·4	+10	7·3	—	—
	66	12·4	+38	7·6	—	—
	71	11·8	+36	7·7	—	—
	73	10·6	Nil	—	5·9	—
	135	24·9	+42	—	5·9	—
	98	16·0	+19	—	6·6	—
	101	19·0	+18	—	6·6	—
	133	33·4	+56	—	—	3·2
	176	30·8	+ 6	—	—	4·1
	200	35·0	+26	—	—	3·3
	158	29·0	+12	—	—	2·8
$Ct=4200$; $t=2$ min. (3 hr. before measurement)	61	10·0	+41	6·3	—	—
	69	10·0	+20	5·1	—	—
	126	22·4	+29	—	7·0	—
	136	20·4	+ 6	—	6·9	—
	177	32·4	+18	—	—	2·9
	207	31·8	Nil	—	—	2·6
$Ct=4080$; $t=8$ min. (19 hr. before measurement)	71·3	9·0	−11	5·7	—	—
	155	16·0	−32	5·2	—	—
	84	10·4	− 6	—	6·1	—
	161	16·6	−32	—	4·3	—
	146	18·6	−19	—	—	2·95
	116	12·9	−32	—	—	3·5
$Ct=4200$; $t=2$ min. (27 hr. before measurement)	34	5·4	+24	7·6	—	—
	30	4·4	+17	7·7	—	—
	78	13·4	+26	—	7·7	—
	54	8·8	+13	—	7·0	—
	104	19·4	+31	—	—	3·0
	86	25·2	+17	—	—	2·1

weight of lung tissue increases following exposure to phosgene causing a relative decrease in the nucleic acid phosphorus of the lungs.

DISCUSSION

The experiments reveal no great change in the metabolism of the lung following exposure to phosgene. If the oedema of the lung were due to the poisoning of enzymes concerned in carbohydrate metabolism some change should have been observed. Thus, although it may be possible that vesication of the skin by mustard gas is due to poisoning of hexokinase, it seems very improbable that poisoning of this enzyme or of any of the other enzymes concerned with glycolysis in the lung, occurs on exposure to phosgene.

SUMMARY

1. The lungs of young adult mice are sufficiently small to allow of measurement of metabolism without mincing. Mean values of 7·3 for Q_{O_2} and 3·0 for $Q_G^{N_2}$ were found.

2. No great changes in the metabolism of the lung were observed on exposure of mice to phosgene, and it therefore appears unlikely that the oedema of the lung is due to poisoning of enzymes concerned with glycolysis or respiration in the lung.

We are indebted to the Chief Scientist of the Ministry of Supply for permission to publish these results.

Table 3. *Weight of lung and nucleic acid phosphorus in the lungs of normal and phosgene-poisoned mice*

Details of mouse				Nucleic acid P of lung	
Exposure	Wt. (g.)	Wet wt. of lung (mg.)	Wt. of lung as % of body wt.	(As % of lung wt.)	(As % of body wt.)
None	19	133	0·705	0·115	0·00081
	23	156	0·678	0·109	0·00073
	21	152	0·722	0·114	0·00082
	19·5	148	0·759	0·108	0·00076
	22	141	0·640	0·107	0·00068
	20	161	0·806	0·108	0·00087
	21·8	146	0·650	0·112	0·00075
	22·5	196	0·871	0·110	0·00096
	18	100	0·556	0·098	0·00055
	19·5	135	0·693	0·108	0·00073
	20·5	143	0·698	0·111	0·00077
	19·5	122	0·626	0·115	0·00072
Phosgene $Ct = 3480$ (4½ hr. before estimation)	20·5	312	1·52	0·062	0·00094
	22	301	1·37	0·069	0·00094
	23	310	1·35	0·064	0·00086
	19·5	314	1·61	0·058	0·00094
	19	296	1·56	0·059	0·00092
	21	320	1·52	0·064	0·00098
Phosgene $Ct = 2240$ (22 hr. before)	19	277	1·46	0·051	0·00075
	20	213	1·06	0·081	0·00086
	21	269	1·28	0·069	0·00088
	20	305	1·52	0·051	0·00078
	19	164	0·864	0·087	0·00075
	20·5	185	0·903	0·087	0·00078

REFERENCES

Boyland, E., McDonald, F. F. & Rumens, M. J. (1946). *Brit. J. Pharmacol.* **1**, 81.

Berenblum, I., Chain, E. & Heatley, N. G. (1939). *Biochem. J.* **33**, 68.

Laser, H. (1932). *Biochem. Z.* **248**, 9.

The Electrophoretic Analysis of Protein Extracts from Striated Rabbit Muscle

2. DENATURATION IN ACETATE BUFFERS

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In a previous paper (Jacob, 1947) some interrelations between the classical muscle proteins (myogen, myoalbumin, myosin, globulin *X*) and the electrophoretic components of various extracts of striated rabbit muscle were established. In order to complete the characterization of components, the analysis of one of these muscle extracts in acetate buffers of decreasing pH is described here. This is necessary since the isoelectric points of several components lie in the pH range covered by acetate buffers, and in an acid medium, most of the muscle proteins are denatured without flocculating (Finn, 1932; Weber, 1934; Bate Smith, 1937). These new observations are of interest in the study of denaturation processes.

EXPERIMENTAL

The electrophoretic technique and the procedures for the extraction and preparation of muscle extracts have been described in a previous paper (Jacob, 1947). The extraction solution used here contained 0·048 M-Na_2HPO_4 and 0·006 M-NaH_2PO_4 (pH = 7·7, $\mu = 0{\cdot}15$); the dialysis solutions, which also served as supernatant in the electrophoretic experiments, contained 0·1 M-sodium acetate, 0·05 M-NaCl

and a variable amount of acetic acid. Their pH varied from 3·62 to 5·92, with ionic strength constant at $\mu = 0{\cdot}15$.

The extracts were dialyzed for 24–70 hr., and some protein was found to precipitate. When the extract was centrifuged after 3 days' dialysis, the volume of precipitate was greater the lower the pH of dialysis. When the extract was centrifuged several times during dialysis, it was possible to collect a precipitate during the first 36–60 hr., in the pH range 5·0–5·9; whereas, at any lower pH, after the 8–24 hr. precipitates had been removed, no further precipitation occurred.

The total amount of precipitate was larger and its formation more rapid the lower the pH. The dialysis time was sufficient to avoid further precipitation during electrophoresis, though at pH 5·9 failures were frequent. The dialyzed extracts became less turbid as the pH decreased, and below pH 5·0 they were almost limpid.

When dialyzed extracts were left at room temperature, a further precipitate appeared; the quantity and rapidity of formation of this increased with the fall of pH, thus illustrating the progressive instability of the extracts. Between pH 3·9 and 3·6, the extracts were extremely viscous and had a tendency to form a thixotropic gel, even when kept at 1°.

As in the preceding paper, pH measurements were made at room temperature. To calculate the pH at 0°, the values were corrected by 0·025 pH unit, according to Harned & Ehlers (1932).

The relative amounts of the different components were obtained from the peak areas after correcting for significant superimposed δ and ϵ effects. Depending on the pH, there were two possibilities: (1) the δ and ϵ effects were superimposed on a protein peak located at the extremity of the pattern (here this peak is *h*); (2) the δ and ϵ effects were superimposed on one or more peaks located in the centre of the pattern (here these effects shift progressively from *l* to *h* as the pH decreases).

The first case is often recorded in the literature, and the values obtained from the descending pattern are usually adopted. By this means, the amount obtained for the extreme peak represents in fact the total of the amounts which can be attributed to that peak and to the ϵ effect, which is generally neglected. In our experiments, when the ϵ effect is dissociated from the protein peaks, it represents on the average 4% of the total surface of the diagram. Consequently, the values obtained from the descending pattern were corrected, by subtracting from the apparent amount of the extreme peak the amount due to the ϵ effect (4% of the total surface), and sharing this amount between the peaks, in proportion to their relative magnitude (this is in conformity with the theoretical developments of Longsworth, 1942).

When the δ and ϵ effects are superimposed on one or more median peaks, it is sufficient to consider that each of the patterns, the anode and cathode, contains ascending, descending and median peaks. The first step is to determine, on both diagrams, the relative amounts of the descending peaks. The difference between 100% and the total of these relative amounts represents the percentage to be attributed to the median peak and to the ϵ effect. It is then easy to eliminate the ϵ effect by the same procedure as in the first case.

It would have been a great advantage, if it had been possible, to correct the ascending values, in order to measure asymmetries depending on causes other than δ and ϵ effects. Unfortunately, the precision of the measurements was considerably reduced by the poor separation of one ascending peak at any pH higher than 5·0, and by the excessive sharpness of the gradient of another ascending peak at lower pH values.

Finally, it was impossible to establish whether the relative amounts of the different peaks altered during the course of electrophoresis.

RESULTS

Description of the patterns obtained in acetate buffers

At pH 5·9–5·8, the patterns obtained in acetate buffers differ only slightly from those obtained (Jacob, 1947) at neighbouring pH values in phosphate buffers. The separation into three main groups again occurs (Fig. 1). The first group consists of the peak *n*, which separates early, and of the two components *l* and *m*, which only appear after prolonged experiments at lower ionic strength ($\mu = 0{\cdot}10$). The second group contains its usual components k_1, k_2 and *j*. Between groups II and III lies a small quantity of protein which is not easily divided between these two groups. For convenience it was called *sp* (space). The third group consists of a single peak, unimpaired by any turbidity. Probably the turbid protein *i* has vanished from it, and this peak represents the component *h* to which it approximates in amount.

The relative concentrations of the components are not widely different, whether the dialysis buffer is acetate or phosphate (Table 1).

As the pH decreases, the patterns change progressively (Fig. 1). The relative area of peak *n* increases regularly from 18% (pH 5·9) to 99% (pH 3·64) (Table 1, col. *C*). This increase in concentration is not so regular when expressed as mg. N/ml. because of non-systematic variations (up to 20% at least) in the total protein-N concentration of the dialyzed extracts. If these errors are taken into account, the change referred to the amount of N is also regular (Fig. 2). The concentration of peak *n* rises progressively to 1·2, 2·0, 2·4, 3·5 mg. N/ml., values which are much higher than the concentration of the substance *n* in extracts dialyzed against phosphate buffer (0·72–0·90 mg. N/ml.; Jacob, 1947). This peak thus represents not only the substance *n*, but also other proteins which behaved as separate entities in phosphate buffer. Consequently another designation *C* was adopted for this peak.

The shape of *C* differs in the anode (descending) and cathode (ascending) patterns. Up to pH 4·0, the characteristics of this asymmetry are classical (Tiselius & Horsfall, 1939; Longsworth & MacInnes, 1940). The ascending boundary is sharp and very homogeneous; the descending boundary is wide, and its 'buffer' side is steeper than its 'protein' side; this becomes more typical and almost excessive, as the pH decreases. Below pH 4·0, the descending

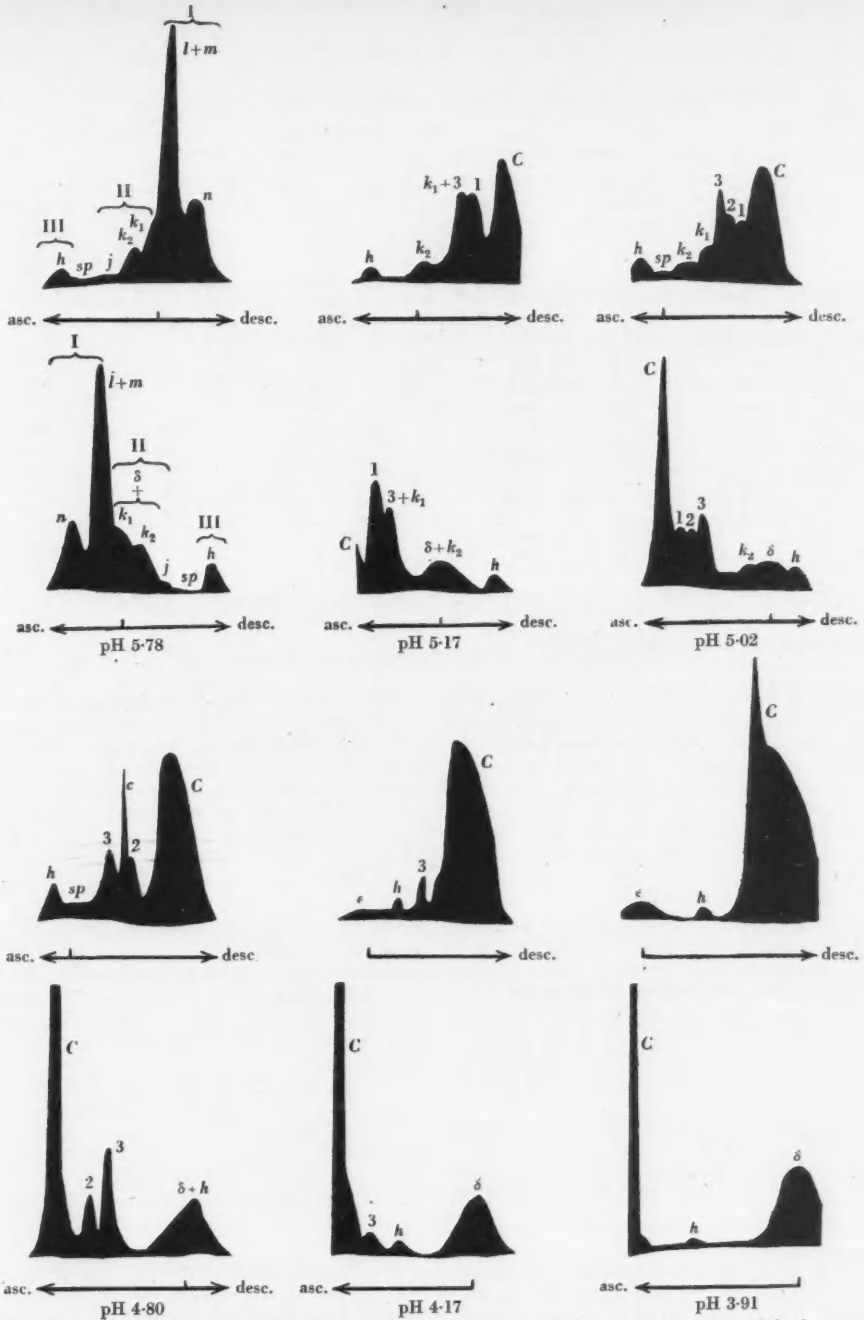

Fig. 1. Electrophoretic patterns of muscle extracts dialyzed against acetate buffers, $\mu = 0{\cdot}15$. First and third rows: anodic patterns; second and fourth rows: cathodic patterns; asc., ascending part; desc., descending part from the origin *O*. Details of runs were as follows:

pH	V./cm.	Sec. of electrophoresis	pH	V./cm.	Sec. of electrophoresis
5·78	4·07	18,840	4·80	4·33	20,160
5·17	4·38	19,080	4·17	4·00	16,920
5·02	4·00	20,460	3·91	4·27	18,300

Table 1. *Composition of muscle extracts dialyzed in acetate buffers*

No.	pH	Protein-N (mg./ml.)	Relative amounts of the components from corrected descending values (%) C	$l+m$	k_1	k_2+j	sp	h
1	5·92	1·80*	19·5	54·5	8·0†	9·0†	3·0	6·0
P‡	5·79	—	19·5	55·0	18·0		2·0	5·5
2	5·78	—	23·5	[55·0]	[7·5]	6·0†	2·5	5·5
3	5·62	—	33·0	51·0	5·0†	4·5†	2·5	4·0
4	5·61	3·61	35·5	48·0	5·0†	5·5†	2·0	4·0
5	5·45	2·52	34·5	50·0	5·0†	4·0†	2·5†	4·0
6	5·36	3·02	40·5	43·0	6·0	4·5†	2·0†	4·0
7	5·33	3·05	39·5	43·5	6·0	3·5†	1·5†	6·0
8	5·22	3·87	44·5	39·5	6·0	4·0†	2·0†	4·0
9	5·17	2·69	46·0	[40·5]	[4·5]	3·0†	2·0†	4·0
10	5·05	4·32	48·0	40·0	4·0	2·0†	2·0†	4·0
11	5·02	2·86	50·0	32·5	7·0	2·5†	1·5†	6·5
12	4·91	2·71	57·5	34·5		3·0†		5·0
13	4·85	4·00	62·0	31·0		2·0†		5·0†
14	4·80	3·51	66·5	27·0		2·0†		4·5†
15	4·69	—	69·5	24·0		2·5		4·0†
16	4·45	—	77·5	18·5		—		4·0†
17	4·34	3·66	81·0	14·5		1·0		3·5
18	4·17	3·80	[86·5]	[9·5]		—		4·0
19	3·91	3·92	98·0	—		—		2·0
20	3·64	—	99·0	—		—		1·0

* Artificially reduced value, not accounted for in Fig. 2.
† Indicates the peaks to which the ϵ effect was added.
P‡ Experiment with phosphate buffer.
[ ] Indicates the peaks whose separation was not satisfactory.

boundary splits in two and then several streaks form, whereas the ascending boundary apparently becomes more homogeneous. This suggests that the progressive spreading of the anode curve from pH 5·0 to 4·0 is a prelude to its cleavage at all lower pH values.

The peak common to l and m remains homogeneous down to pH 5·30 and its relative amount decreases from 54·5 to 43 %. From pH 5·2 to 4·45, this boundary undergoes several successive scissions. The patterns become complex and are characterized by asymmetries which even involve the number of peaks. Simultaneously, the total amount corresponding to these peaks falls as low as 18·5 %.

Between pH 5·20 and 5·05, $l+m$ divides into two components whose relative amounts differ in the anode and cathode compartments (peak 1 (the faster) and peak 3); there is nothing to facilitate identification of these peaks with the components l and m.

In the neighbourhood of pH 5, an additional peak (peak 2) appears between 1 and 3; 1 becomes gradually smaller and simultaneously migrates closer to C and, mainly on cathodic patterns, its final inclusion in C can clearly be followed.

From pH 4·9 downwards, the asymmetry between the two patterns becomes more complex. In the anode pattern a component (c) appears whose gradient is relatively very sharp, and no corresponding peak forms in the cathode pattern.

Below pH 4·45, the group is simplified by the fusion with C of the residue of the streaks developed from the cleavage of boundary $l+m$.

The peaks of group II disappear progressively. Component k_1 separates less from peak 3, mainly on the cathode diagram, and below pH 5·00 it merges into peak 3. Component k_2 is sharply defined down to pH 4·9. It decreases in amount from pH 5·30 to 4·9 and at pH 4·8, no trace of it remains. Component j is marked by a slight inflexion on the side of k_2, which gets less and less distinguishable. Component h is, of all components, the one which undergoes the least alteration. Down to pH 4·1, its quantity measured from the descending boundary does not diminish, and it shows no peculiar characteristic. Below pH 4·0, it decreases, but at pH 3·64, it is still evident as a small inflexion. It is the only one among the peaks occurring at pH 5·9 which does not completely disappear, or is not completely absorbed by C.

Down to pH 4·85, the diagrams are mixed (Jacob, 1947), i.e. both the anode and cathode diagrams include a δ and an ϵ effect. The most obvious of these, the cathode δ effect, shifts progressively from l to sp between pH 5·9 and 4·9; it then reaches streak h, which it lifts, as far as pH 4·4, and then separates completely. The ϵ effect shows very clearly in the anode diagrams below pH 4·4.

Finally, the slight turbidity is no longer associated with groups II or III as in the phosphate experiments, but migrates with group I from pH 5·6 downwards.

In short, with the progressive fall of pH, the proteins tend to migrate as a single boundary (C).

The other components, with the exception of *h*, finally disappear either because they are incorporated in *C*, or because they precipitate in the course of the preliminary dialysis (which is probably the case for *j* and k_2). Before disappearing, some components undergo alterations (in the case of $l+m$ and k_2).

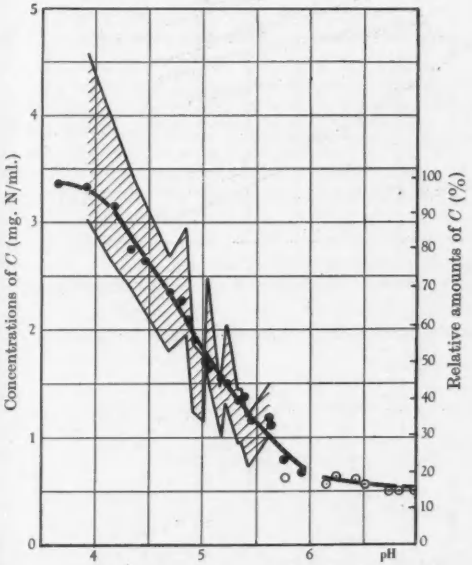

Fig. 2. Concentrations (in mg. N/ml., left scale) and relative amounts (in %, right scale) of boundary *C* as a function of the pH. The relation of the two scales was established with 100% as an average of the total amounts of protein N in the dialyzed extracts (3·4 mg. N/ml.). The streaked area is obtained by joining the points corresponding to the concentrations increased and lowered by 20%. ● Relative amounts of boundary *C* (acetate buffer); ○ relative amounts of boundary *n* (phosphate buffer – values previously published (Jacob, 1947)). The corresponding pH values have since been corrected according to Bates & Acree (1945).

Among more than thirty experiments, a single one—executed at pH 5·15—gave aberrant patterns in which the normal components maintained their individuality; the reason for this divergence could not be found.

Complex formation between components

The progressive incorporation of the protein components of the extracts into one electrophoretic peak can have two interpretations: either (*a*) the mobilities of different components become so similar in some zones of pH that no electrophoretic separation is possible under the conditions of the experiment; (the resulting peak has no special implication) or (*b*) a complex is formed between two or more proteins, and migrates with a characteristic mobility, distinct from those of the original proteins.

The first possibility seems likely, since the mobility curves of several components finally coincide with the curve of *C* (Figs. 3, 4), but this cannot wholly account for the behaviour of *C*, since before the fusions occur the amount of *C* increases considerably (from 18% at pH 5·9 to 57·5% at pH 4·90). Further, the contribution of each protein does not account quantitatively for the increase of *C* (for instance, from pH 4·90 to 4·40, the amount of *C* rises from 57·5 to 77·5%, whereas the amount of peak 1 is, at the most, 7–8%); finally, the increase of *C* is not irregular but almost continuous (Fig. 2). Probably *C* is primarily a complex composed of *n* and a portion of the other components.

Interactions are likely to occur between complex *C* and several other components, at all pH values lower than 4·9. Indeed, we notice the existence on the anode pattern of a peak (*c*), which does not appear on the cathode pattern.

A plausible interpretation is the following (according to Longsworth & MacInnes, 1942). On the anode (descending) side, the components 1, 2 and 3 migrate in a medium devoid of *C*. They have a tendency to re-form the complex *C*, but in the absence of *n* which has also left the medium, the aggregation product migrates more slowly than *C* and is represented by peak *c*. The mobility of *c* is not widely different from the average mobilities of 1, 2 and 3 at pH 4·8 and 4·9, and of the average mobilities of 2 and 3 at all lower pH's when 1 has been completely absorbed by *C*. This does not occur on the ascending side where components 1, 2 and 3 migrate in a medium containing complex *C*. On the contrary, one would expect complex *C* to dissociate when it migrates alone; this probably occurs, but the errors involved in the area measurements of the ascending peaks make it difficult to establish.

Further, important asymmetries occur between the ascending and descending mobilities. It is, in general, very difficult to estimate the part to be attributed to the 'classical' asymmetry, and to the 'interaction' asymmetry, but this is not the case for peak *h* below its isoelectric point. For this component, the 'classical' asymmetry is almost negligible owing to its low concentration (Jacob, 1947). This is true down to pH 4·67, but below this pH, i.e. when *h* begins to form part of the complex, the asymmetry becomes considerable (Fig. 4). Undoubtedly interactions occur which cannot otherwise be detected, owing to the difficulty of measuring exactly a peak of such small area.

It should be mentioned that dilution of the extract causes a diminution in the amount of complex *C* (for instance, the extract no. 9, diluted once with acetate buffer of an identical pH contains only 39% of *C* instead of 46% and likewise, the extract no. 14, diluted in the same manner contains only 55% instead of 66%). This agrees with some observations of Longsworth, Cannan & MacInnes (1940),

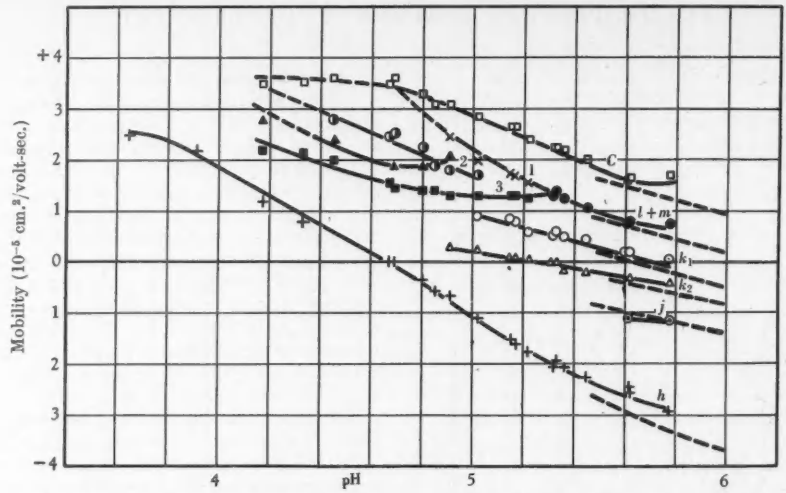

Fig. 3. Descending pH-mobility curves of the electrophoretic components of muscle extracts in acetate buffers of $\mu = 0{\cdot}15$.
□ = C, ● = $l+m$, × = 1, ◑ = 2, ■ = 3, ▲ = c, ○ = k_1, △ = k_2, ⊙ = j, + = h.
The dashed curves (- - - -) were obtained with phosphate buffers of the same ionic strength (Jacob, 1947; for the pH, see note of Fig. 2 and text, p. 77).

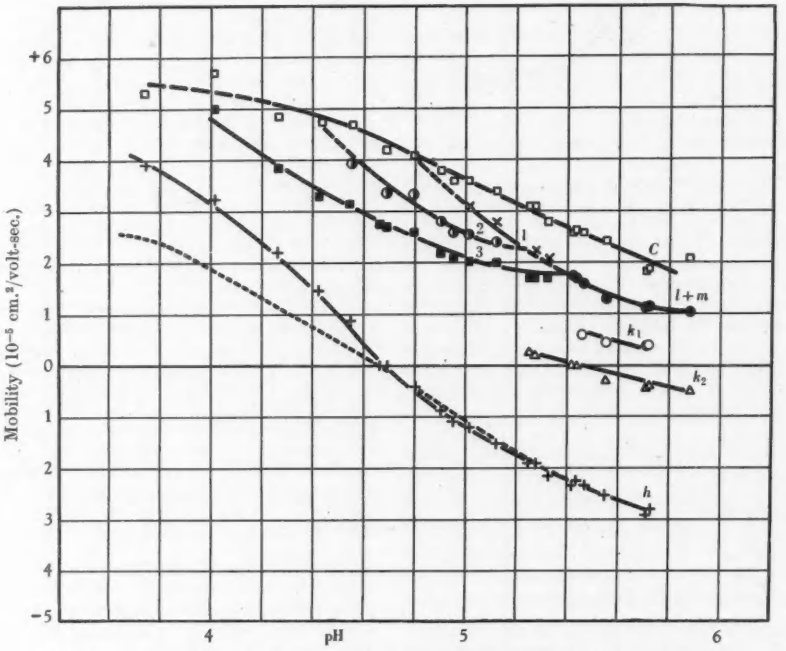

Fig. 4. Ascending pH-mobility curves of the components in acetate buffers of $\mu = 0{\cdot}15$.
□ = C, ● = $l+m$, × = 1, ◑ = 2, ■ = 3, ○ = k_1, △ = k_2, + = h.
The broken (lower) curve (- - -) is the descending curve of h.

that aggregation phenomena between proteins are less marked when the protein concentration is low.

Interactions are not likely to be artefacts of the moving boundary method (Perlmann & Kaufman, 1945; Svensson, 1946), for dilution does not result in the disappearance of the asymmetric peak *c*, or a significant reduction of the mobility asymmetries of the various components. It may be concluded that the increase in the area of *C* is due mainly to the formation of a complex. Probably this component interacts with the other components present.

Contrary to facts mentioned by Moyer & Moyer (1940) for rabbit serum proteins, and by Longsworth (1941) for ovalbumin, the pH mobility curves of muscle proteins—in phosphate and acetate buffers—do not diverge noticeably in the common pH zone (Fig. 3). It is worth mentioning that this agreement is found only if the pH values are corrected to 0°, which can be done by using the data of Bates & Acree (1945) for phosphate buffers, and Harned & Ehlers (1932) for acetate buffers.

phosphate was prolonged (two days, three renewals of the outer liquid), in order to ensure equilibrium between buffer and protein. Its duration does not exceed the limits which were established empirically for the conservation of extracts dialyzed against phosphate buffer alone (Jacob, 1947). In these experiments the identity of the components remaining in solution can be decided from their mobilities, which are very close to those of the components in extracts which were not dialyzed initially against acetate. Very often, they are slightly higher, which may be accidental; there is no evidence of a definite alkaline shift.

The results of the concentration measurements are given in Table 2. If these results are compared with the values obtained for the same extract, after acetate dialysis alone (Table 1), it appears that the loss of concentration is approximately equal to the amount of the complex *C*; and that the amounts of each of the components remaining in solution are close to the amounts of the corresponding peaks of

Table 2. *Composition of muscle extracts after two dialyses*

					Relative amounts† of protein				
					Soluble part				
No.*	pH of acetate buffer	pH of phosphate buffer	mg. N/ml. after 2nd dialysis	Ppt.‡	*M* (+*n*)	*l*	k_1	$k_2 j + sp$	*h*
§	—	7·10–7·80	4·0±1·0	0	47·5	24	±7	±12	±5
7	5·33	7·63	1·70	44	18·5	25	5	3	4·5
13	4·85	7·67	1·86	53·5	19	19	2	2	4·5
17	4·34	7·56	0·66	82	4	10·5	1·5		2
19	3·91	7·52	0·32	92	4·0		1·0		3

* This number indicates the extract of Table 1, a part of which was subjected to the second dialysis.

† Relative amounts are calculated relative to the N concentration of the extract after the first dialysis. They can thus be compared directly with the amounts of corresponding experiments in Table 1.

‡ Includes precipitation and experimental losses etc. (see text).

§ Average results of experiments made after a single phosphate dialysis without any detectable preliminary denaturation (Jacob, 1947).

Recovery of native (or 'reversibly' denatured) proteins from the extracts treated with acetate buffers

The native components *h*, *sp*, *k*, *l*, *m* and *n* of the muscle extracts are soluble in a solution containing 0·032 M-Na_2HPO_4, 0·04 M-NaH_2PO_4 and 0·5 M-NaCl, pH 7·60, $\mu = 0{\cdot}15$ (Jacob, 1947).

If the extracts are first dialyzed against an acetate buffer, centrifuged and then dialyzed a second time against this phosphate solution, a precipitate forms in less than 1 hr. The quantity of protein remaining in solution after removal of the precipitate decreases as the pH of the acetate solution is lowered. The loss is negligible at pH 5·9, but reaches almost 100% when the pH is 3·64. There is thus a progressive denaturation of the muscle proteins in acetate buffers as the pH falls. This confirms the results of Finn (1932), Weber (1934) and Bate Smith (1937).

The proteins which remain in solution have been examined electrophoretically. Dialysis against the extract after acetate dialysis alone. This suggests that *C* is the aggregation product of the denatured proteins which precipitate during a second dialysis.

However, the parallelism is not absolute; the loss of concentration represents both the elimination of the precipitate and accidental losses in the course of the second dialysis (osmotic dilution, etc.); it should thus always be greater than the amount of *C*. Yet the opposite is observed in Exps. 13 and 19, and in Exps. 7 and 17 where the loss of concentration is greater than the amount of the complex, this difference being balanced by a fall in the concentration of those peaks which are not yet much involved in the process of progressive aggregation (e.g. *k*, *j*, *sp* and *h* in Exp. 7; *h* in Exp. 7). Presumably the other components remaining in solution are present in a relatively excessive concentration. Consequently, the complex *C* in so far as its real amount is estimated from the descending pattern, is not entirely precipitated. It would thus include, besides the denatured proteins—insoluble in a phosphate buffer—a small proportion of native protein or of denatured proteins soluble in a phosphate buffer.

DISCUSSION

The main object of this study was to characterize the electrophoretic components of extracts from rabbit muscle. In the previous paper (Jacob, 1947), the following isoelectric points were determined: n, 6·75+0·12; m, 6·20+0·12; l, 6·05+0·12; k_1, 5·50+0·12 (for the correction+0·12 see Bates & Acree, 1945). Using acetate buffer, this list is completed with the values: h, 4·65; k_2, 5·25; k_1, 5·70; the isoelectric point of j lies between 4·7 and 5·10.

The isoelectric point of component h is the lowest of all those we have observed. This fraction is not denatured at least down to pH 4·85 (Table 2). This characteristic could not be used to prepare h directly in a purified state; at all pH's lower than 4·90, the elimination of denatured proteins leaves in solution a substantial proportion of proteins other than h. At pH 3·62, the undenatured proteins are very dilute, and electrophoresis of the solution at pH 7·6 has for that reason given no reliable information.

This group of characteristics confirms the identification of peak h with the myoalbumin of Bate Smith. The only divergence relates to the position of the isoelectric point: 3·5 according to Bate Smith (1937), 4·65 on our curve. This divergence may be due either to the use of different buffers at different temperatures, or to the fact that Bate Smith's myoalbumin was modified considerably and adsorbed on quartz particles.

The isoelectric point of h is almost identical with that of serum albumin, but h is not identical with serum albumin, however, as has been shown in a preceding paper (Jacob, 1945).

Similarly, the isoelectric points of j and k_2 differ from those given by Green (1945) for the phosphorylases a and b, which seem to constitute, wholly or partly, these two components respectively (compare the curves and diagrams given by Green with our results, obtained in a phosphate buffer). The divergence is probably due to the fact that this author was compelled to extrapolate more extensively than the present data require.

In group I, the water-soluble part of which corresponds with Weber's myogen (Jacob, 1947) component l is not denatured at least down to pH 5·33. By ultracentrifugation, Gralén (1939) has found that the crystalline myogen A of Baranowski (1939) was stable to a pH of about 5·0; l would correspond mainly to this myogen, which is confirmed by unpublished experiments by Distèche in this laboratory.

The evolution of component n is strange; from pH 5·8 it is wholly included in the denaturation complex. All the evidence seems to indicate that the protein n is a centre of formation for the complex C. It is not impossible that, in a phosphate buffer, n is already a denatured protein (or a protein aggregate) which has undergone an alkaline shift (cf. Neurath, Greenstein, Putman & Erickson, 1944). This hardly seems probable, for in a phosphate buffer, the amount of n does not change much with the pH (Fig. 2). On the other hand, when an extract is denatured in a phosphate buffer by ageing, there is no increase in n, but the turbid matter collects at the level of group II, a group which migrates with a mobility approximately equal to the average mobility of all muscle components (cf. an analogous phenomenon for serum, Davis, Hollaender & Greenstein, 1942). The evolution of n gives rise to many hypotheses which it will be possible to examine only when purified muscle proteins are isolated and studied.

The denaturation of a mixture of muscle proteins in acetate buffers is mainly characterized by the formation of a complex. A similar phenomenon occurs in heated serum (Van der Scheer, Wyckoff & Clarke, 1941; Kleczkowski, 1941), in serum irradiated with ultra-violet rays (Davis *et al.* 1942) and when a mixture of casein and antibody is heated (Krejci, Jennings & Smith, 1942). It is generally admitted that this aggregation is a phenomenon distinct from denaturation proper; it would be made possible by the emergence of new reactive groups in a denatured protein molecule.

The points which characterize the formation of the complex C are: (1) its continuous increase with decrease of pH, (2) the probable existence of interactions between the complex and certain other proteins of the mixture.

If the interpretation given of these interactions is really accurate, complex formation would be an equilibrium reaction, the constant of which would be determined essentially by the pH (or by the concentration of acetic acid). The condition of equilibrium would also be influenced by the concentrations of the proteins involved. This phenomenon can be interpreted either (*a*) as an equilibrium between the original and the denatured forms of a protein (the latter form being the only one liable to enter into the formation of the complex); or (*b*) as a probability of aggregation of molecules which would all be denatured, but to varying degrees, according to the pH (for instance, at a given pH, all molecules are unfolded more than at the immediately higher pH, but less than at the immediately lower pH). This leads us to the hypothesis of Neurath, Cooper & Erickson (1942). The facts presented have, besides, a certain analogy with the experiments on 'reversible' denaturation by urea and guanidine, described by the authors. Indeed, both in Neurath's experiments and in ours the elimination of the denaturing agent brings about the partial precipitation of proteins. This process differentiates the proteins into irreversibly denatured proteins and others which remain soluble, of which in our case we cannot be certain whether they are identical with or slightly different from the original proteins.

We cannot develop any further this idea of equilibrium, and the very numerous problems which it suggests and could solve. The mixture analyzed here is too complex and too poorly defined.

Lastly, the aggregation complex is altered at low pH; this is shown on patterns such as Fig. 1, pH 3·9, which resemble diagrams given by Longsworth (1941) for the acid denaturation of ovalbumin. The interpretation of such asymmetrical diagrams is still unelucidated, but the similarity stresses the analogy of the action of acetic acid, in high concentration, on such widely different proteins as crystalline ovalbumin and a complex of modified muscle proteins.

SUMMARY

1. Protein extracts of rabbit muscles have been submitted to electrophoresis, in buffers containing 0·1M-Na acetate, 0·05M-NaCl and various concentrations of acetic acid (total $\mu = 0{\cdot}15$, pH varying between 3·6 and 5·9). As the pH decreases, the patterns diverge more and more from the type of pattern obtained with the same extracts in a phosphate buffer.

2. The analysis of the changes reveals the formation of an aggregation complex and the probability of interactions between this complex and other components of the mixture.

3. If the extracts dialyzed against acetate buffer are then subjected to a second dialysis against a phosphate solution at pH 7·6, a precipitate appears. The latter increases the lower the pH of the acetate buffer, and its amount corresponds approximately to the amount of the aggregation complex formed at that pH.

4. The relation between the electrophoretic components of our extracts and the 'classical' muscle proteins are discussed.

5. The meaning of the aggregation complex (denaturation complex) and its evolution in the course of electrophoresis are interpreted, as far as the great complexity of the observed mixture allows.

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REFERENCES

Bates, R. G. & Acree, S. F. (1945). *Bur. Stand. J. Res., Wash.*, **34**, 373.
Baranowski, T. (1939). *C.R. Soc. Biol., Paris*, **130**, 1182.
Davis, B. D., Hollaender, A. & Greenstein, J. P. (1942). *J. biol. Chem.* **146**, 663.
Finn, D. B. (1932). *Proc. Roy. Soc.* B, **111**, 395.
Gralén, N. (1939). *Biochem. J.* **33**, 1342.
Green, A. A. (1945). *J. biol. Chem.* **158**, 315.
Harned, H. & Ehlers, R. W. (1932). *J. Amer. chem. Soc.* **54**, 1350.
Jacob, J. (1945). *Bull. Soc. Sci. Liége*, **4**, 242.
Jacob, J. J. C. (1947). *Biochem. J.* **41**, 83.
Kleczkowski, A. (1941). *Brit. J. exp. Path.* **22**, 188.
Krejci, L. E., Jennings, R. K. & Smith, L. D. (1942). *J. Immunol.* **45**, 111.
Longsworth, L. G. (1941). *Ann. N.Y. Acad. Sci.* **41**, 267.
Longsworth, L. G. (1942). *Chem. Rev.* **30**, 323.
Longsworth, L. G., Cannan, R. K. & MacInnes, D. A. (1940). *J. Amer. chem. Soc.* **62**, 2580.
Longsworth, L. G. & MacInnes, D. A. (1940). *J. Amer. chem. Soc.* **62**, 705.
Longsworth, L. G. & MacInnes, D. A. (1942). *J. gen. Physiol.* **25**, 507.
Moyer, L. S. & Moyer, E. Z. (1940). *J. biol. Chem.* **132**, 373.
Neurath, H., Cooper, G. R. & Erickson, J. O. (1942). *J. biol. Chem.* **142**, 249.
Neurath, H., Greenstein, J. P., Putman, F. W. & Erickson, J. O. (1944). *Chem. Rev.* **34**, 157.
Perlmann, G. E. & Kaufman, D. (1945). *J. Amer. chem. Soc.* **67**, 638.
Smith, E. C. Bate (1937). *Proc. Roy. Soc.* B, **124**, 136.
Svensson, H. (1946). *Ark. Kemi Min. Geol.* **22** A, 1.
Tiselius, A. & Horsfall, F. L. (1939). *Ark. Kemi Min. Geol.* **13** A, 1.
Van der Scheer, J., Wyckoff, R. & Clarke, F. L. (1941). *J. Immunol.* **40**, 39.
Weber, H. H. (1934). *Ergebn. Physiol.* **36**, 109.

The Conversion of DL-3:5-Diiodo-4-hydroxyphenyllactic Acid into an Analogue of Thyroxine

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In extending the work of Ludwig & von Mutzenbecher (1939) on the iodination of proteins under mildly alkaline conditions, leading to the production of thyroxine within the protein molecule, von Mutzenbecher (1939) illustrated the probable mechanism involved by showing that 3:5-diiodotyrosine, on standing at comparable pH, was converted, in small yield, into thyroxine. The subsequent literature dealing with the conversion of free diiodotyrosine into thyroxine has been cited by Harington & Pitt Rivers (1945; see also Reineke & Turner, 1946), who investigated the conditions of the reaction in some detail and postulated an oxidative mechanism, involving iodine, which they suggest may be of biological significance.

There appears to be no report indicating whether or not derivatives of diiodotyrosine have been found to undergo spontaneous transformation to the corresponding analogues of thyroxine under similar conditions. Of special interest in this connexion is 3:5-diiodo-4-hydroxyphenyllactic acid, which Foster & Gutman (1930) isolated from the urine of rabbits in receipt of large doses of diiodotyrosine. Its identity was ascertained by analysis and by a consideration of its chemical behaviour, but was not confirmed by synthesis.

The present communication is a brief report of the synthesis of DL-3:5-diiodo-4-hydroxyphenyllactic acid, and of the results of preliminary experiments designed to effect its conversion into DL-3:5-diiodo-4-(3′:5′-diiodo-4′-hydroxy)phenoxyphenyllactic acid.

EXPERIMENTAL

Synthesis of DL-3:5-*diiodo*-4-*hydroxyphenyllactic acid*

The 4-hydroxyphenyllactic acid required for iodination was obtained from the azlactone of 4-acetoxy-α-acetamidocinnamic acid by two-stage hydrolysis to the corresponding pyruvic acid, followed by reduction.

Azlactone of 4-*acetoxy*-α-*acetamidocinnamic acid.* Acetylglycine (5·85 g.), 4-hydroxybenzaldehyde (6·1 g.), acetic anhydride (13·4) and fused sodium acetate (3 g.) were refluxed for 1 hr., cooled, and placed overnight at 0°. The mass of yellow-brown crystals was broken up with a rod after the addition of 12 ml. of water. The solid was filtered, washed with ice water and dried *in vacuo* over KOH. Yield (crude): 8·7 g. (85%). The crude material melted at 128°.

4-*Acetoxy*-α-*acetamidocinnamic acid.* The crude azlactone (5 g.) was boiled with water (200 ml.) for 10 min. and the hot mixture filtered with gentle suction. The filtrate on cooling deposited yellow crystals which were filtered, washed with water and dried at 110°. Yield: 2·6 g. (48%); m.p. 224°. A specimen recrystallized from water (Norite) gave: N, 5·30%. (Calc. for $C_{13}H_{13}O_5N$, 5·32%.) The hydrolysis of the azlactone with water gave better yields than the acetone-water mixture recommended for the corresponding derivative of benzaldehyde (*Organic Syntheses*, **2**, 519).

4-*Hydroxyphenylpyruvic acid.* The acetamido acid (2·63 g.) was refluxed for 4 hr. in an all-glass apparatus with 2N-HCl (35 ml.) and placed overnight at 0°. The crystals of the keto acid were filtered and dried *in vacuo*. The crude material (1·0 g. (56%); m.p. 210°; Neubauer (1909), has recorded m.p. 220° for purified material) was reduced soon after preparation as some specimens became pasty on standing.

DL-4-*Hydroxyphenyllactic acid.* The crude keto acid (2 g.) was shaken for 10 min. at room temperature with sodium amalgam (1·25%; 130 g.) and water (25 ml.). The separated and acidified (2N-HCl) solution was extracted thrice with an equal volume of ether. The dried ethereal extract deposited an oil on evaporation, which rapidly solidified to a mass of colourless needles. Yield: 1·3 g. (64%). Recrystallized from ether-light petroleum, the substance melted at 120°, resolidified and then gave a m.p. of 135°. (Erlenmeyer & Lipp (1883) record m.p. 115–122° and 139–140° respectively.) On checking, at a later date, this specimen was found to have m.p. 137–139°, without prior change. Found (after drying at 80°): C, 59·4; H, 5·27. (Calc. for $C_9H_{10}O_4$: C, 59·3; H, 5·49%.)

DL-3:5-*Diiodo*-4-*hydroxyphenyllactic acid.* The method of iodination used, adapted from that described by Zeynek (1929) for the preparation of 3:5-diiodotyrosine, has been found suitable in this laboratory for the production of a range of diiodophenols. Iodination with iodine monochloride, as utilized by Block & Powell (1943) for the formation of diiodotyrosine, did not yield a satisfactory product.

A solution of 4-hydroxyphenyllactic acid (1·22 g.) in 2N-H_2SO_4 (13·7 ml.) and 10% (w/v) KI (29·4 ml.) was stirred rapidly at 0° with an electric stirrer. Iodic acid (10% (w/v); 15·6 ml.) was then added dropwise from a funnel, followed by glycine (2 g.) in water (30 ml.) to act as a buffer. 2N-NaOH (16 ml.) was then added over 20 min. to adjust the pH to 8 (approx.). Stirring was continued at 0° for 1 hr. and the mixture then allowed to come to room temperature, when it was acidified with 2N-HCl (6 ml.) and extracted thrice with 50 ml. of ether. The ethereal solution was washed with the minimum amount of aqueous $NaHSO_3$ to remove excess iodine and then with water. The oily residue, after removal of the ether from the still moist solution, was extracted successively with 40, 20 and 10 ml. of boiling water. On cooling in ice, 1·25 g. (44%) of colourless needles were deposited. The crystals lost water at 75° and then melted at 164° (Foster & Gutman (1930) report 73–75° and 158–159° respectively for their only

partly racemic material). The acid is readily soluble in organic solvents and sparingly soluble in cold water. When boiled in water at pH 7·5 the solution darkened appreciably in colour; after 2 hr. the pH had dropped to 5·5 and it was not found possible to isolate any of the original material. The apparent destruction of the lactic acid under these conditions is in accord with the findings for diiodotyrosine (Harington & Pitt Rivers, 1945; Dickinson & Trikojus, 1947). A specimen was purified for analysis by recrystallization from water and drying at 100° *in vacuo*. Found: C, 24·6; H, 2·08; I, 59·1. (Calc. for $C_9H_8O_4I_2$: C, 24·9; H, 1·84; I, 58·5%.)

Experiments on the transformation of 3:5-*diiodo*-4-*hydroxyphenyllactic acid*

The iodinated lactic acid was incubated at pH (initial) 8·8–9·8 and at temperatures of 37 or 55°, with and without the passage of oxygen. The best yield of transformation product was obtained by the following procedure; a careful comparison with other sets of conditions was not, however, carried out.

The lactic acid (1·0 g.) was dissolved in NaOH (0·1N; 23 ml.) and 1N-NaOH added to bring the pH to 8·8. The solution was incubated at 37° for 7 days, a stream of oxygen being provided by passage through a sintered-glass gas distributor. On the 2nd and 4th days *n*-butanol (5 ml.) was added. At the conclusion of the incubation period, sufficient 10N-NaOH was added to the reaction product to make the aqueous phase normal with respect to this reagent. Further butanol was added to equalize the volumes. The butanolic extract was shaken with an equal volume of 2N-NaOH and then evaporated to dryness under diminished pressure. The residue was acidified with 2N-acetic acid and the precipitate purified by dissolving in hot 50% (v/v) acetic acid (1 ml.), filtering from a small residue of amorphous material and then suddenly chilling the hot solution. The yield at this stage was 28 mg. while 350 mg. of pure starting material was recovered. On recrystallization of a portion from dilute acetic acid, an equivalent total recovery of 18 mg. was obtained, the nett yield (about 3%) of this analytically pure material being thus comparable to those obtained by Harington & Pitt Rivers (1945) and Reineke & Turner (1946) in the conversion of diiodotyrosine into thyroxine. Found (after drying at 100°): I, 65·2%. (Calc. for $C_{15}H_{10}O_5I_4$: I, 65·35%.) It crystallized in colourless prisms, m.p. 176–177° (with prior softening), and gave an intense reddish purple colour when treated in *n*-butanol with aqueous alkali and diazotized *N'*-diethylsulphanilamide, a reagent developed in this laboratory for the estimation of thyroxine (Winikoff & Trikojus, 1948). Under appropriate conditions 3:5-diiodo-4-hydroxyphenyllactic acid and 3:5-diiodotyrosine do not give a positive reaction, so that the reagent provides a convenient test in transformations and other studies in this series. The m.p. (173°) recorded for the ketonic acid analogous with thyroxine (Canzanelli, Guild & Harington, 1935) is close to that of our compound, while the iodine value would not differentiate between the two formulae. Although a pure specimen of the keto acid was not available, an examination of the infra-red absorption of our compound was undertaken. A solution in morpholine, 5 mg./ml., was examined in the region 5·50–6·50 μ and only one band, at 6·3 μ, characteristic of the carboxylate ion, was found to be present. By contrast, two strong absorption bands, at 5·89 and 6·3 μ, were observed for α-ketoglutaric acid under similar conditions. The first of these is exactly in the correct position for a ketonic carbonyl group. We have, therefore, assumed that the transformation product of 3:5-diiodo-4-hydroxyphenyllactic acid obtained in these experiments is the lactic acid analogue of thyroxine.

SUMMARY

DL-3:5-Diiodo-4-hydroxyphenyllactic acid has been synthesized and its behaviour studied under conditions similar to those which have been found to result in the conversion of diiodotyrosine to thyroxine. A new compound, considered to be the lactic acid analogue of thyroxine, was isolated in a nett yield of about 3%.

While one of us (V. M. T.) was abroad, laboratory facilities, enabling the identification of the transformation product of 3:5-diiodo-4-hydroxyphenyllactic acid to be carried out, were kindly provided by the Director, National Institute for Medical Research, London (Dr C. R. Harington, F.R.S.) and Dr W. Engels of the Research Department, Merck and Co., Inc., Rahway, New Jersey. We are further indebted to Mrs R. V. Pitt Rivers of the former institution for the iodine assay and to Dr N. R. Trenner of Merck and Co. for the infra-red examination and report.

REFERENCES

Block, P., Jr. & Powell, G. (1943). *J. Amer. chem. Soc.* **65**, 1430.
Canzanelli, A., Guild, R. & Harington, C. R. (1935). *Biochem. J.* **29**, 1617.
Dickinson, J. & Trikojus, V. M. (1947). *Aust. J. Sci.* **9**, 152.
Erlenmeyer, E. & Lipp, A. (1883). *Liebigs Ann.* **219**, 226.
Foster, G. L. & Gutman, A. B. (1930). *J. biol. Chem.* **87**, 289.
Harington, C. R. & Rivers, R. V. Pitt (1945). *Biochem. J.* **39**, 157.
Ludwig, W. & von Mutzenbecher, P. (1939). *Hoppe-Seyl. Z.* **258**, 195.
von Mutzenbecher, P. (1939). *Hoppe-Seyl. Z.* **261**, 253.
Neubauer, O. (1909). *Chem. Zbl.* **2**, 51.
Reineke, E. P. & Turner, C. W. (1946). *J. biol. Chem.* **162**, 369.
Winikoff, D. & Trikojus, V. M. (1948). *Biochem. J.* **42** (in the Press).
Zeynek, E. (1929). *Z. ges. exp. Med.* **68**, 482.

The Inhibition of Thiol Enzymes by Lachrymators*

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This work was undertaken in order to throw light upon the biochemical mechanism of the lachrymatory effect of certain war gases. Owing to the relationship of one of these, ethyl iodoacetate, to the well known biochemical inhibitor, iodoacetic acid, the study was first directed towards enzymes known to be inhibited by this compound, namely, certain thiol enzymes, or enzymes whose activity appears to depend on the integrity of sulphydryl groups. Iodoacetic acid inhibits muscle glycolysis and yeast fermentation by acting on the triose-phosphate dehydrogenase and the yeast alcohol dehydrogenase (Dixon, 1937; Rapkine, 1938; Adler, Gunther & von Euler, 1938). Dickens (1933), and Quastel (1933), showed that iodoacetic acid reacted with thiol groups in cysteine, reduced glutathione and proteins. Since then the list of thiol enzymes has been growing steadily (see Barron & Singer (1945) and Hellermann (1939) for reviews). The following work shows that the lachrymators react with simple thiol groups, that they inhibit almost all the enzymes generally considered to be of a thiol nature, that these enzymes can be protected by free sulphydryl groups, and that most of the enzymes not affected by the lachrymators are believed on other evidence not to contain essential thiol groups. It is suggested that inhibition by the lachrymators may be used as a test for thiol enzymes in general.

METHODS

The lachrymators tested were as follows: ethyl iodoacetate, methyl iodoacetate, bromoacetophenone, chloroacetophenone, bromobenzyl cyanide and chloropicrin. They were dissolved (0·25 M) in ethylene glycol monoethyl ether and in all cases the same amount of solvent was added to the control. The experiments were carried out at a temperature of 37° unless otherwise stated.

RESULTS

Reaction with enzymes

Succinic dehydrogenase

The system used for testing this enzyme was prepared from heart muscle, following the method used by Straub (1939) for the preparation of diaphorase, as far as the precipitation at pH 4·6. This precipitate was suspended in a small amount of phosphate buffer pH 7·4, giving a highly active preparation containing all the enzymes necessary for the oxidation of succinic acid by oxygen, namely, succinic dehydrogenase, flavoprotein, cytochrome *c* and cytochrome oxidase. The enzyme was tested in Barcroft manometers, using about 0·1 ml. of enzyme suspension in 1 ml. phosphate buffer pH 7·6 and 1·6 ml. water. KOH papers were used to absorb any CO_2 formed. Sodium succinate (0·4 ml., 20 mg./ml.) was tipped from a Keilin tube at time 0 into the right-hand flask. The lachrymator was tipped in from another Keilin tube either with the substrate or at a given time before it.

Fig. 1 shows various results obtained. The action of the poison is progressive, so that the inhibitory effect depends on both the concentration of the poison and the time of incubation. The rate of inhibition falls off as the reaction between the enzyme and lachrymator proceeds, an equilibrium finally being reached if excess poison is not used.

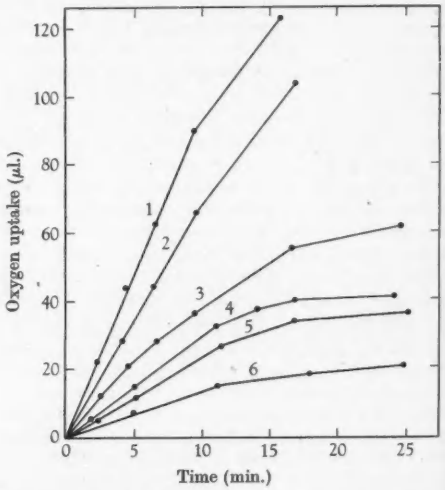

Fig. 1. The effect of lachrymators on the oxygen uptake of the succinic dehydrogenase—cytochrome system. Curve 1, control. Curve 2, M/10,000 ethyl iodoacetate. Curve 3, M/3000 ethyl iodoacetate. Curve 4, M/10,000 bromoacetophenone. Curve 5, M/15,000 chloropicrin. Curve 6, M/10,000 chloropicrin. The lachrymators were added with the substrate at time 0, and the curves show how the inhibition increases with time.

* This work was carried out in 1940–1 under the direction and supervision of Dr M. Dixon for the Ministry of Supply. It was reported to the Ministry in 1941 (Mackworth, 1941). The author is indebted to the Chief Scientist of the Ministry of Supply for permission to publish this paper.

Ethyl iodoacetate takes longer to reach equilibrium than the other lachrymators. The inhibitory reaction also depends on the presence of any protective substances, especially denatured enzyme, which inevitably occur in varying concentrations in such an enzyme preparation. Therefore any comparative work must be carried out on the same preparation and as far as possible at the same time. Table 1 shows the effects of the different lachrymators on the succinic dehydrogenase—cytochrome system.

Table 1. *Inhibition of succinic dehydrogenase—cytochrome system by lachrymators*

Poison	Conc.	Incubation (min.)	Inhibition (%)
Bromobenzyl cyanide	M/3,000	0	70
		5	85
Ethyl iodoacetate	M/1,500	10	97
	M/3,000	10	90
	M/6,000	10	85
	M/12,000	10	60
Chloropicrin	M/15,000	15	72
	M/50,000	30	68
	M/100,000	15	40
Chloroacetophenone	M/3,000	0	20
		5	42
		20	82

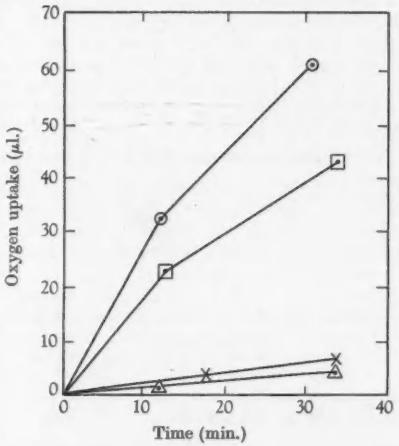

Fig. 2. The effect of lachrymators on succinic dehydrogenase. The cytochrome system has been inhibited by KCN, and methylene blue used in its place. ⊙—⊙ Control; ⊡—⊡ M/10,000 ethyl iodoacetate; ×—× M/10,000 bromoacetophenone; △—△ M/10,000 chloropicrin.

By adding 0·3 ml. 0·01 M-neutralized KCN and 0·3 ml. of 0·5% methylene blue the cytochrome system is inhibited, and the succinic dehydrogenase remains the only active enzyme in the system, methylene blue catalyzing the reaction with oxygen. Fig. 2 shows that the effect of the lachrymators on this system is just as great as before, thus making it clear that the lachrymators attack the dehydrogenase itself. Experiments on the oxidation of *p*-phenylenediamine by the cytochrome oxidase in the preparation show that this enzyme is not affected by M/1500 methyl iodoacetate.

Experiments on protection. It was found that addition of cysteine or reduced glutathione before adding the lachrymator protected the enzyme from the expected inhibition. Considerable excess, about nine times as much thiol as lachrymator, was necessary for complete protection. This may be due partly to the greater affinity of the lachrymator for the enzyme than for the simple thiol, as suggested below, but also it is known that the simple thiols are rapidly oxidized by the enzyme system here used.

Table 2 shows the results, the inhibition being calculated as far as possible from the initial rate over the first 5 min. The enzyme was incubated with the

Table 2. *Protection of succinic dehydrogenase by cysteine and reduced glutathione from the effects of the lachrymators*

Poison	Conc.	Conc. of GSH	Initial inhibition (%)
Ethyl iodoacetate	M/5000	0	70
		M/2000	40
Bromoacetophenone	M/9000	0	85
		M/3000	70
		M/2000	58
		M/1000	30
		Conc. of cysteine	
Chloropicrin	M/9000	0	95
		M/1000	20
Bromoacetophenone	M/4500	0	85
		M/1000	25
Bromoacetophenone	M/9000	0	95
		M/3000	75
		M/1000	0

poison, with or without the thiol compound, for 20 min., so that the rate of increase in inhibition during the period of the experiment was usually fairly slow (cf. Fig. 3).

This protection could be used to determine accurately the inhibition produced by the lachrymator after a given time, because by tipping in excess cysteine or glutathione the poisoning could be arrested at any required point. For instance Fig. 3 shows the effect of adding excess glutathione to the succinic system after incubation for 20 min. with M/3000 ethyl iodoacetate. In the flask in which glutathione (GSH) has not been added the inhibition continues to increase, whilst in the flask in which GSH is added with the substrate, the inhibition is arrested, and remains at that level at

which it was in both experiments when the substrate was added. These results show that although GSH protects the enzyme, there is no reversal, and in fact no evidence of reversal was ever obtained in working with lachrymators on succinic dehydrogenase.

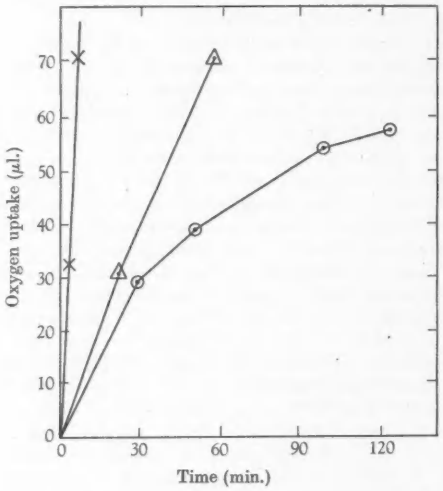

Fig. 3. Protection of succinic dehydrogenase from the effects of ethyl iodoacetate by reduced glutathione. ×—× Control; △—△ after 20 min. incubation with M/3000 ethyl iodoacetate, excess GSH is tipped in with the substrate at time 0; ⊙—⊙ after incubation with ethyl iodoacetate the substrate alone is tipped in at time 0.

Since it thus appears that the poisoning is arrested immediately the thiol compound is added, it is possible to obtain figures for the relation between the time of action of the poison and the activity of the enzyme. Table 3 shows the results of adding cysteine (M/1500) at varying times after the addition of bromoacetophenone to succinic dehydrogenase. It appears that the rate of poisoning is not uniform, but falls off until no further poisoning can be obtained over a considerable period of time. This is the result that might be expected if the rate of inhibition depended on the concentrations of both the enzyme and the poison, and if both were destroyed during the reaction. However, destruction of the poison by something other than the enzyme would produce similar results, and it is clear from the slightly varying levels of inhibition produced by the poison on different preparations containing the same amounts of active enzyme that such destruction does occur. Only experiments with pure enzyme could clarify this point.

Table 3. *Effect of terminating bromoacetophenone poisoning of succinic dehydrogenase with cysteine*

Conc. of bromoacetophenone	Time of incubation before cysteine added (min.)	Activity remaining (%)
M/62,000: Exp. I	2	82
	5	45
	10	22
Exp. II	4	50
	8	45
	16	29
M/37,000	2	35
	5	17
	7	17

The most interesting protection is that by boiled enzyme. It was found that 0·2 ml. of boiled inactive enzyme would completely protect an equal amount of the active enzyme from poisoning by M/9000 bromoacetophenone, which without the boiled enzyme produced an inhibition of 95%.

Choline dehydrogenase

The enzyme was prepared from rat liver (Mann, Woodward & Quastel, 1938) by grinding in a mortar with phosphate buffer pH 7·4. The suspension was then homogenized in the Booth-Green bacterial mill for 15–20 min., centrifuged, and the fluid dialyzed against running tap-water for 1–2 hr. This preparation keeps for about 2 days at 0°. The oxidation of choline using 1 ml. of enzyme was followed either by oxygen uptake in Barcroft manometers or by methylene blue reduction in evacuated Thunberg tubes. The preparation contained all the factors necessary for the oxidation of choline by oxygen.

Table 4 shows the effects of the lachrymators after 20 min. incubation. As with succinic dehydrogenase, the inhibition was progressive, and the results show the initial inhibition when the substrate was first added, measured over the first 5–10 min.

Table 4. *The effects of lachrymators on choline dehydrogenase*

Conc. of poison		Inhibition (%)
A. In Barcroft manometers:		
Chloroacetophenone	M/2500	65
	M/1500	95
Bromobenzyl cyanide	M/2500	80
Bromoacetophenone	M/2500	90
	M/4500	90*
Ethyl iodoacetate	M/1500	—
(No incubation)		20
(20 min. incubation)		80
B. In Thunberg tubes:		
Bromobenzyl cyanide	M/6000	50
	M/3000	60
	M/2000	75

* Obtained with a specially purified preparation.

Table 5 shows the protection afforded by cysteine against M/1500 chloroacetophenone, after 20 min. incubation with the poison, as measured in Barcroft manometers. In this experiment it was found that 0·2 ml. of boiled enzyme was sufficient to give complete protection.

Table 5. *Protection of choline dehydrogenase by cysteine against* M/1500 *chloroacetophenone*

Conc. of cysteine	Inhibition (%)
0	95
M/500	82
M/250	45

Yeast alcohol dehydrogenase

The enzyme was prepared by the first step of the method of Negelein & Wulff (1937), which gives a very active preparation. Juice prepared from dried yeast was heated, cooled and a half volume of acetone was added to the centrifugal fluid. The precipitate was discarded and a further half volume of acetone was added. This precipitate, containing the enzyme, was dried over H_2SO_4 and dissolved in water for use. It was tested in Thunberg tubes with methylene blue. In order to complete the system, cozymase and diaphorase were added and semicarbazide to remove the acetaldehyde formed. The enzyme was incubated with the lachrymators for 15 min. before the ethanol and dye were tipped from the stopper.

Table 6 shows that the lachrymators have a considerably greater effect on the enzyme than has iodoacetate.

Papain

British Drug Houses papain was used, and activated by adding 30 mg. cysteine in solution at pH 4·8 to 5 ml. of 0·5% papain solution. The substrate used was fresh milk plus 0·2 vol. of 0·2M-acetate buffer, the mixture having a pH of 4·6.

The reaction with ethyl iodoacetate is complicated by the necessity of activating papain with cysteine which would react with the iodoacetate; while if the papain and iodoacetate were incubated together before addition of cysteine, it seemed likely, from what is already known about papain, that the thiol group in the enzyme might be masked in a disulphide linkage. This proved to be the case, as the following results show. Activated papain (1 mg.) clotted the milk in 2 min., whereas 10 mg. natural papain were necessary for this clotting time. When incubated with 0·01 M-ethyl iodoacetate before activation and then treated with M/60 cysteine, 20 mg. of papain were necessary for the same speed of clotting, whereas if first activated with 0·02 M-cysteine, incubated with 0·02 M-ethyl iodoacetate and then treated with 0·04 M-cysteine, no activity remained.

This showed that the papain could be regarded as less susceptible to the ethyl iodoacetate before activation, as was to be expected if in the inactive form the thiol group were combined in an S—S linkage.

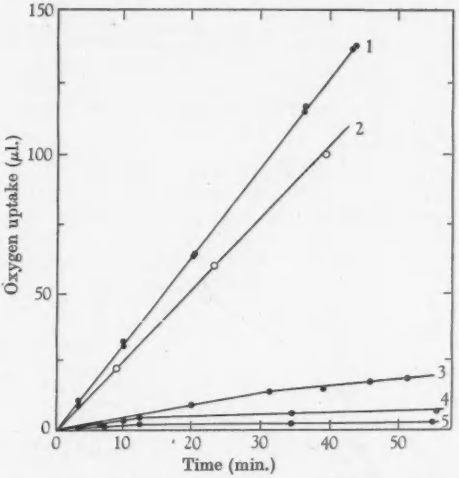

Fig. 4. The effect of the lachrymators on pyruvic oxidase of *Esch. coli*. Incubation 20 min. Curve 1, control and with M/1600 sodium iodoacetate. Curve 2, M/6000 ethyl iodoacetate. Curve 3, M/1600 methyl iodoacetate. Curve 4, M/3000 chloropicrin. Curve 5, M/3000 bromoacetophenone.

Pyruvic oxidase

The enzyme was prepared from *Esch. coli* by the method of Still (1941) and tested in Barcroft manometers as described by him. Fig. 4 shows the results.

Table 6. *The effect of lachrymators on yeast alcohol dehydrogenase*

	Time of reduction of methylene blue		Inhibition (%)
	min.	sec.	
Control	1	45	—
+ Na iodoacetate (M/3000)	2	35	60
+ ethyl iodoacetate (M/3000)	>60	—	100
+ ethyl iodoacetate (M/6000)	8	—	85
+ bromoacetophenone (M/3000)	>60	—	100
+ chloropicrin (M/3000)	>60	—	100
+ bromobenzyl cyanide (M/3000)	>60	—	100
+ chloroacetophenone (M/3000)	8	—	85

Triosephosphate dehydrogenase

A 5% suspension of muscle acetone powder, prepared as described by Green, Needham & Dewan (1937), was centrifuged and the soluble portion used as the source of the enzyme and of the other enzymes necessary for the testing reaction. The substrate was added in the form of hexosediphosphate solution, which is rapidly converted into triosephosphate by the zymohexase present in the extract. This latter enzyme is not inhibited by the lachrymators. The reaction was carried out in Barcroft manometers in bicarbonate solution equilibrated with a gas mixture containing 5% CO_2 in N_2. Coenzyme I was added as hydrogen carrier, and pyruvate to accept the hydrogen removed from the triosephosphate. The phosphoglyceric acid formed was measured by the CO_2 evolution.

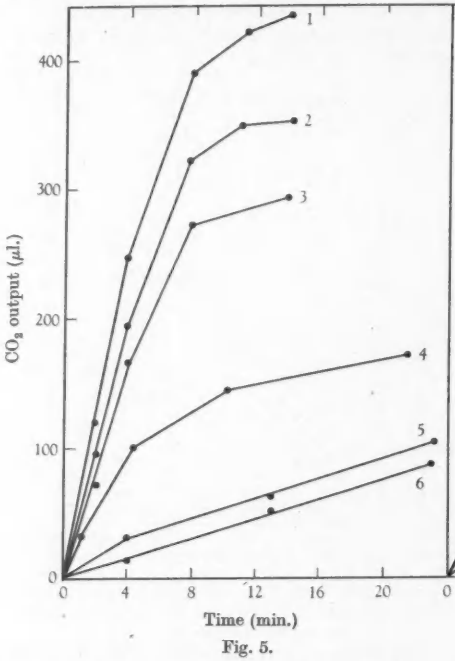

Fig. 5.

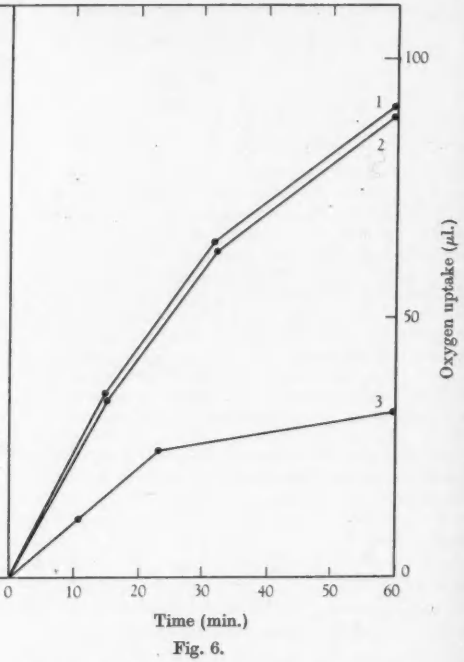

Fig. 6.

Fig. 5. The effect of lachrymators and sodium iodoacetate on triosephosphate dehydrogenase. Incubation 20 min. Curve 1, control for curves 2 and 3. Curve 2, M/3000 chloropicrin. Curve 3, M/3000 bromacetophenone. Curve 4, control for curves 5 and 6. Curve 5, M/3000 sodium iodoacetate. Curve 6, M/3000 ethyl iodoacetate.

Fig. 6. The effect of methyl iodoacetate and sodium iodoacetate on xanthine oxidase. Incubation 15 min. Curve 1, control. Curve 2, M/660 sodium iodoacetate. Curve 3, M/660 methyl iodoacetate.

Table 7. *The effect of lachrymators on triose phosphate dehydrogenase*

Poison	Conc.	Inhibition (%)
Na iodoacetate	M/3000	90
	M/5000	60
Methyl iodoacetate	M/3000	90
	M/5000	50
Ethyl iodoacetate	M/3000	90
Chloropicrin	M/3000	20
Bromobenzyl cyanide	M/2000	45
Bromoacetophenone	M/2000	50
	M/3000	30
Chloroacetophenone	M/1000	30

Table 8. *The effect of the lachrymators on xanthine oxidase, tested in Thunberg tubes*

Poison	Time of reduction of methylene blue min.	sec.	Inhibition (%)
None	—	50	—
Bromobenzyl cyanide (M/1200)	10	—	93
Bromoacetophenone (M/625)	8	—	85
(M/2400)	4	30	80
Chloropicrin (M/1200)	1	45	66
(M/500)	3	—	75

This enzyme differs from all the other thiol enzymes in being quite as sensitive to the iodoacetate ion as to the lachrymators. Fig. 5 shows the effects of M/3000 iodoacetate and some of the lachrymators. The two experiments recorded here have different controls owing to the varying activity of the muscle powder. Because the control reaction is not linear, the degree of inhibition is not readily determined, but Table 7 shows the initial inhibitions obtained after 20 min. incubation with the lachrymators.

Xanthine oxidase

The enzyme was used in the form of a whey preparation as described by Dixon & Kodama (1926) and the oxidation of hypoxanthine by oxygen followed in Barcroft manometers. Portions of 1·6 ml. of a 10% solution of the enzyme were added to both flasks and also 0·05 ml. of an active preparation of catalase, to destroy any hydrogen peroxide formed. Hypoxanthine (1 mg.) was added from a Keilin tube at time 0. The enzyme was incubated with methyl iodoacetate for 15 min. before adding the substrate.

Fig. 6 shows the effect of M/660 methyl iodoacetate as compared with the same concentration of sodium iodoacetate.

The other lachrymators were tested by the Thunberg tube method, using methylene blue. Table 8 shows the results.

Cholinesterase

The enzyme was prepared in a purified form from horse serum by the method of Stedman & Stedman (1935). The hydrolysis of acetylcholine was followed in Barcroft manometers, by measuring the amount of CO_2 set free from bicarbonate-Ringer solution by the free acid formed. After 15 min. incubation with the lachrymators 3 mg. of acetylcholine in neutralized solution were tipped in.

Table 9 shows the effects of the lachrymators on this enzyme after 30 min. incubation. It is clear that it is much less sensitive than any of the previous enzymes. A crude extract of rabbit's brain, prepared by grinding the brain with sand and pressing through muslin, was also tested, but the brain enzyme was found to be unaffected by M/1250 bromoacetophenone.

Table 9. *The effect of lachrymators on cholinesterase from horse serum*

Poison	Conc.	Inhibition (%)
Bromobenzyl cyanide	M/900	25
	M/365	70
Bromoacetophenone	M/1250	Nil
	M/365	60
Chloropicrin	M/365	Nil
Ethyl iodoacetate	M/600	Nil

Urease

Urease tablets (0·1 g.) were extracted with 10 ml. phosphate buffer pH 7·4 and the insoluble part filtered off. The activity was measured by the method of Sumner (1926), slightly modified. 1 ml. of a solution containing 5·4 g. K_2HPO_4, 4·25 g. KH_2PO_4 and 0·15 g. urea/100 ml. was added to 1 ml. of the enzyme solution, and after standing at room temperature for the required time, the ammonia content of the samples was estimated with Nessler solution.

It was found that after 15 min. incubation with M/400 ethyl iodoacetate 50% inhibition was produced, while M/600 bromoacetophenone gave 80% inhibition. The enzyme was therefore not as sensitive as might be expected from its known thiol nature, but this might have been due to impurities in the solution.

Carboxylase

The CO_2 liberated by dried yeast extract acting on pyruvate was measured in Barcroft manometers. An inhibition of about 20% was obtained by incubating the yeast with M/1500 ethyl iodoacetate for 15 min. before adding the substrate.

Glyoxalase

The enzyme was prepared from horse liver and tested by the method of Lohmann (1932). The methylglyoxal was placed in the flasks of Barcroft manometers with the enzyme, the glutathione being tipped in from Keilin tubes.

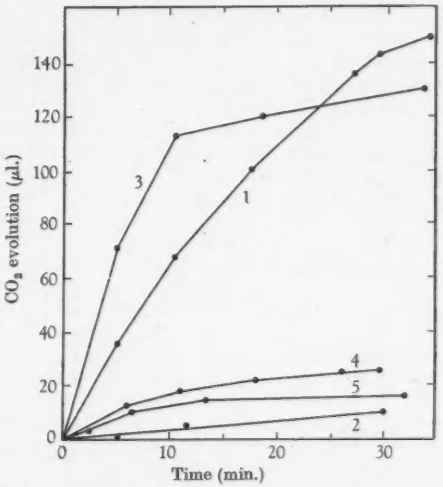

Fig. 7. The effect of the lachrymators on glyoxalase and its coenzyme, reduced glutathione. Curve 1, enzyme and 50 μg. GSH. Curve 2, 50 μg. GSH incubated with 300 μg. bromoacetophenone before addition to the enzyme. Curve 3, M/2300 bromoacetophenone incubated with the enzyme, before 400 μg. GSH were added. Curve 4, 50 μg. GSH incubated with 500 μg. ethyl iodoacetate before being added to the enzyme. Curve 5, 50 μg. GSH incubated with 260 μg. chloropicrin.

Fig. 7 shows the results of treating the enzyme or the coenzyme, reduced glutathione, with lachrymators. In curve 1, 50 μg. of GSH were added to

the enzyme from a Keilin tube at time 0. Curve 2 shows the effect of incubating the GSH in the Keilin tubes with 300 μg. of bromoacetophenone before tipping. The concentration of lachrymator was *c.* M/70 in the Keilin tube and, after tipping, M/2300 in the flask. In curve 3, M/2300 bromoacetophenone was incubated with the enzyme and then 400 μg. of GSH were tipped in. While the incubation of the lachrymator with the GSH has almost completely destroyed the activity, treatment of the enzyme appears not to inhibit. This is due to the fact that the rate depends partly on the concentration of GSH. Curves 4 and 5 show the destruction of GSH by incubating 50 μg. GSH with 500 μg. ethyl iodoacetate and 260 μg. chloropicrin respectively. It is clear that all three lachrymators destroy GSH.

Metabolism

Muscle respiration

Pigeon-breast muscle or rat-leg muscle was minced in a Latapie mincer with the grinding die removed, and suitable amounts (usually about 200 mg.) were weighed directly into the manometer flasks. The solution contained 1 mg. of succinate to stabilize the respiration. Any non-cyclic oxidation of succinate would be finished before readings were taken. The method was that of Stare & Baumann (1936). The lachrymators were tipped in during the experiment so that the rates of O_2 uptake of each portion of tissue both before and after the addition of the poison were known. Portions of chopped rat-leg muscle were similarly treated.

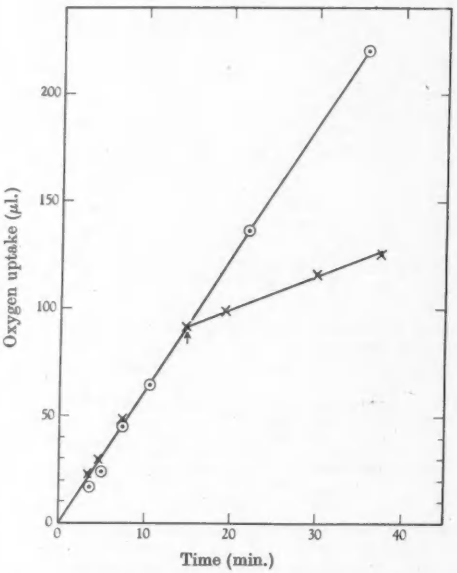

Fig. 8. The inhibition of rat-leg muscle respiration by bromoacetophenone. ⊙—⊙ Control; ×—× as control at the beginning, M/2000 bromoacetophenone being added at the arrow.

Fig. 8 shows the effects on the respiration of rat-leg muscle of adding 0·0005M-bromoacetophenone at the point indicated by the arrow. While the control continued at the rate already established, the muscle treated with the lachrymator immediately ceased to respire. Similar results were obtained with pigeon-breast muscle, of which the respiration was almost completely inhibited by M/1500 ethyl iodoacetate.

Yeast fermentation

The fermentation of glucose by baker's yeast was followed in Barcroft manometers by measuring the CO_2 produced in the absence of O_2, the flasks being filled with N_2 freed from O_2 by passing over heated copper. The lachrymators were tipped in from Keilin tubes so that accurate measure of the time of incubation could be obtained.

All the lachrymators, except chloropicrin, inhibit glucose fermentation by yeast, the inhibition being progressive and irreversible. The inhibition produced by 0·0002M-ethyl iodoacetate, bromoacetophenone or bromobenzyl cyanide becomes almost complete in about 20 min. Chloroacetophenone produces about 60% inhibition in 40 min. The inhibition produced by 0·0002M-chloropicrin reached 30% in 5 min., but did not increase during the next 20 min. The reason for this will be explained in the next section.

Yeast respiration in glucose

The oxygen uptake of baker's yeast was measured in Barcroft manometers, KOH papers being used to absorb the CO_2 formed. 17 mg. of glucose were used as substrate although the respiration would normally continue without added glucose. In order to measure carbohydrate respiration solely the yeast was starved for 24 hr., when the residual respiration without added glucose was very small.

Table 10 shows the inhibition produced after 30 min. incubation, the initial rate of respiration at this time being determined. The inhibition of respiration was progressive, except with chloropicrin. When starved yeast was treated with M/750 methyl iodoacetate, it was found that the poison had no effect on the small oxygen uptake (30 μl. in 90 min.) which occurred without the addition of a substrate, but when 17 mg. of glucose were added, the great increase in respiration (200 μl. in 15 min.) which took place in the control was completely absent in the poisoned yeast, whose respiration remained at the same level as the control yeast without added glucose.

Table 10. *The effect of the lachrymators on yeast (glucose) respiration*

(Conc. poison, M/6000; incubation 30 min.)

Poison	Inhibition (%)
Chloropicrin	40
Chloroacetophenone	70
Ethyl iodoacetate	82
Bromobenzyl cyanide	88
Bromoacetophenone	95

Ethyl iodoacetate, incubation 10 min.

Conc.	Inhibition (%)
M/1,500	85
M/3,000	80
M/6,000	75
M/12,000	65
M/24,000	55
M/48,000	35

It will be noticed from this table that chloropicrin, in marked contrast to its effect on other enzymes, has only a very slight effect on yeast. The reason for this appears to be that yeast can destroy chloropicrin; when treated with 0·001 M-chloropicrin, the initial inhibition of 60% is no longer shown after washing on the centrifuge, and the poisoned yeast becomes as active as the control. Similar treatment of ethyl iodoacetate-poisoned yeast produces no reactivation. Moreover, if M/750 chloropicrin is incubated with yeast for 2 hr. and then the solution is added to the succinic dehydrogenase system, no inhibition at all is obtained, showing that the chloropicrin must have been completely destroyed. Chloropicrin incubated without yeast—and bromoacetophenone incubated with yeast—both gave complete inhibition of the dehydrogenase system. This is the only case found in dealing with lachrymators in which there is evidence of reversal of the inhibition, and it seems likely that it is due to a special enzyme in the yeast, which can remove the chloropicrin from its combination with the inhibited enzyme.

Table 11 shows the enzymes which have been found to be unaffected by the lachrymators in the concentrations shown.

Table 11. *Enzymes not affected by the lachrymators*

Enzyme	Poison	Conc. tested
Cytochrome oxidase	Methyl iodoacetate	M/1500
Carbonic anhydrase	Bromoacetophenone	M/1500
Diaphorase	Methyl iodoacetate	M/300
D-Amino-acid oxidase	Methyl iodoacetate	M/750
Zymohexase	Methyl iodoacetate	M/1500
Glucose dehydrogenase	Ethyl iodoacetate	M/1500
Lactic dehydrogenase (muscle)	Methyl iodoacetate	M/1500
Malic dehydrogenase	Methyl iodoacetate	M/300

DISCUSSION

The foregoing experiments suggest that the action of the lachrymators on the body may be due to inhibition of thiol enzymes, many of which, such as succinic dehydrogenase and pyruvic oxidase, are known to play an important part in the general metabolism of the body. From an analogy with the iodoacetate ion (Dickens, 1933), it is probable that the lachrymators combine irreversibly with the enzyme, their toxicity being dependent on the halide group. They are far more toxic to almost every enzyme than iodoacetic acid, and can be regarded as reliable thiol reagents, when applied in low concentrations. Triosephosphate dehydrogenase shows a marked exception to this generalization, for it is as sensitive to ionic iodoacetate as to its esters, while the other lachrymators have a smaller effect. The only example observed of reversibility is in the inhibition of yeast by chloropicrin.

On the assumption that the lachrymators react only with the thiol group it is suggested that xanthine oxidase, an enzyme not hitherto known to belong to the thiol class, should be included in this class. It has long been known that this enzyme is very sensitive to oxidation, especially by hydrogen peroxide (Dixon & Kodama, 1926), and recently Mann & Quastel (1946) have shown that it is inhibited by oxygen at 1 atm. They suggest that this might be due to the formation of hydrogen peroxide, but in the experiments shown in this paper the peroxide was destroyed by catalase, so that it is more probable that the oxidase is a true thiol enzyme. This is the only flavoprotein which appears to be sensitive to the lachrymators.

There are many enzymes which are completely insensitive to the lachrymators, such as cytochrome oxidase and lactic dehydrogenase, and all workers are agreed that these enzymes are insensitive to all thiol reagents. There are a few enzymes which are only slightly sensitive, such as cholinesterase. Nachmansohn & Lederer (1939) suggested that this was a thiol enzyme, but they found it no more sensitive to thiol reagents than it is to the lachrymators. Finally there is the considerable class of thiol enzymes, which are very sensitive to lachrymators. Dickens (1946) has shown how these enzymes are sensitive to high oxygen pressures, and among them he includes choline oxidase, shown here to be a thiol enzyme. He finds that D-amino-acid oxidase is not sensitive to high oxygen pressure, nor is it sensitive to the lachrymators, although it is sensitive to arsenicals (Barron & Singer, 1945).

The lachrymators appear to react more readily with proteins containing thiol groups than with simple thiols, such as cysteine. While about nine molecules cysteine/molecule lachrymator are required for complete protection, the denatured

enzyme preparation will give protection in concentrations which contain no appreciable thiol groups demonstrable by the nitroprusside test. The combination with the denatured enzyme preparation must have been much faster than with the active enzyme, since if there had been any appreciable competition some of the latter must have been destroyed, and this protective action might be expected on the theory that denaturation increases the number of reactive thiol groups; denatured egg white, however, although it is known to contain numerous thiol groups, had no protective power.

The experiments with papain support the theory that this enzyme contains a thiol group which in the inactive form is probably present as S—S. When the partially inactive form was treated with ethyl iodoacetate and activated after this treatment, the inhibition was not complete. In contrast to this, when the papain was first activated with cysteine and then poisoned, complete inhibition was obtained, although excess cysteine was added after the poison.

The action of yeast on chloropicrin is of considerable interest. It appears that the yeast is capable of destroying the chloropicrin, and that washing can produce complete reactivation of the yeast. As this is the only case in which reversal has been obtained, experiments might be carried out on other enzymes, to see whether yeast could reactivate them when they have been poisoned with chloropicrin. It suggests that reversal might be a question of finding a reactant with a sufficiently powerful affinity for the lachrymator.

SUMMARY

1. An account is given of the effects of various lachrymators on enzyme systems. It is shown that they inhibit all the enzymes which are generally accepted as thiol enzymes, the inhibition being progressive and irreversible in all cases except that of chloropicrin on yeast.

2. The enzymes can be protected against the inhibition by reduced glutathione, cysteine, or denatured enzyme. A considerable excess of the simple thiols is required for complete protection.

3. It is shown that the lachrymators inhibit the respiration of muscle. They also inhibit equally the fermentation and respiration of glucose by yeast.

4. It is suggested that the lachrymators act like iodoacetate in that they combine irreversibly with thiol groups in the enzyme, but they are much more powerful than iodoacetate.

REFERENCES

Adler, E., Gunther, G. & von Euler, H. (1938). *Skand. Arch. Physiol.* **80**, 1.
Barron, E. S. G. & Singer, J. P. (1945). *J. biol. Chem.* **157**, 221.
Dickens, F. (1933). *Biochem. J.* **27**, 1141.
Dickens, F. (1946). *Biochem. J.* **40**, 171.
Dixon, M. (1937). *Nature, Lond.*, **140**, 806.
Dixon, M. & Kodama, K. (1926). *Biochem. J.* **20**, 1104.
Green, D. E., Needham, D. M. & Dewan, J. G. (1937). *Biochem. J.* **31**, 2327.
Hellermann, L. (1939). *Cold Spr. Harb. Symp. quant. Biol.* **7**, 165.
Lohmann, K. (1932). *Biochem. Z.* **254**, 332.
Mackworth, J. F. (1941). Report to Ministry of Supply by Dixon, no. 4.*
Mann, P. J. G. & Quastel, J. H. (1946). *Biochem. J.* **40**, 143.
Mann, P. J. G., Woodward, H. E. & Quastel, J. H. (1938). *Biochem. J.* **32**, 1024.
Nachmansohn, D. & Lederer, G. (1939). *Bull. Soc. chim. Biol.* **21**, 797.
Negelein, E. & Wulff, H. (1937). *Biochem. Z.* **293**, 351.
Quastel, J. H. (1933). *Biochem. J.* **27**, 1116.
Rapkine, L. (1938). *Biochem. J.* **32**, 1729.
Stare, F. J. & Baumann, C. A. (1936). *Proc. Roy. Soc.* B, **121**, 338.
Stedman, E. & Stedman, E. (1935). *Biochem. J.* **29**, 2563.
Still, J. L. (1941). *Biochem. J.* **35**, 380.
Straub, F. B. (1939). *Biochem. J.* **33**, 789.
Sumner, J. B. (1926). *J. biol. Chem.* **69**, 435.

* Information available on application to Ministry of Supply, London.

The Inhibition of Serum Cholinesterase by Alkyl Fluorophosphonates*

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During the war, a team of workers under Drs Saunders and McCombie prepared a series of fluorophosphonate esters and related compounds (McCombie & Saunders, 1946). Some of them were found to be highly toxic to animals by inhalation and very rapid in action, and to have a powerful myotic action when present in sub-lethal concentrations. In man, exposure to amounts of vapour insufficient to give any sensory effect produced a pupillary contraction and spasm of accommodation lasting several days. It was suggested that the parasympatheticomimetic activity of the fluorophosphonates, like that of eserine, might be due to an inhibition of cholinesterase. The results reported here have made it clear that these esters do in fact act like eserine in poisoning the enzyme, and that this reaction *in vitro* can be used as a test for the pharmacological potency of the esters.

EXPERIMENTAL

Materials and methods

Enzyme. Cholinesterase was prepared from horse serum by the method of Stedman & Stedman (1935), carrying the preparation as far as the dialysis against chloroform water. The method of estimation was essentially that of Ammon (1933). The enzyme solution was placed in the right-hand flask of a Barcroft manometer, in a total volume of 3 ml. 0·2% $NaHCO_3$; the gas phase was 5% CO_2 in N_2. The reaction, carried out at 20°, was started by tipping in a solution containing 2 mg. acetylcholine chloride. The CO_2 output was usually linear until about 100 μl. had been produced. The activity remained remarkably constant for many weeks when the stock enzyme was stored at 0° in presence of a little chloroform. For the inhibition experiments, sufficient enzyme was taken to produce 100–120 μl. CO_2 in 10 min. at 20°.

Poisons. The fluorophosphonates and related compounds were obtained from Dr B. C. Saunders; eserine sulphate was a commercial product (British Drug Houses Ltd.). Since fluorophosphonates slowly hydrolyze in water, stock solutions of these and of eserine were prepared in ethylene glycol monoethyl ether, and diluted with the same solvent so that an addition of 0·01–0·03 ml. to the 3 ml. of enzyme-buffer solution in the manometer vessel gave the required final concentration of poison. The enzyme, buffer and poison were incubated in the manometer vessel for the required time (15 min. in the standard test mentioned below) at 20° before tipping in the substrate from dangling tubes. For the experiments with a short duration of incubation, the poison solution was tipped from a dangling tube and the acetylcholine from a side tube.

The activity of the treated enzyme was compared against that of a control incubated with the solvent alone. Since it was found that the enzyme was unaffected by the solvent, or by incubation at 20° without substrate for a period of some hours, it was not necessary to repeat the control with every set of estimations.

RESULTS

Progress curve of inhibition. In preliminary experiments with di*iso*propyl fluorophosphonate, it was found that concentrations above 10^{-7} M completely inhibited cholinesterase almost instantaneously. With lower concentrations, the inhibition produced varied with the time of incubation. Fig. 1

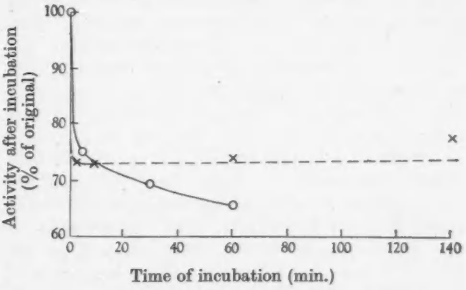


Fig. 1. Progress curve of inhibition of horse serum cholinesterase by eserine and by di*iso*propyl fluorophosphonate, in absence of substrate, at pH 7·4 and 20°. ×—×, 5×10^{-8} M-eserine; ○—○ *c.* 3×10^{-10} M-di*iso*propyl fluorophosphonate.

shows the inhibition of cholinesterase by di*iso*propyl fluorophosphonate, and by a comparable amount of eserine, after varying times. The action of eserine reaches a maximum within 5 min., while the inhibition by fluorophosphonate is initially less rapid, but is progressive and ultimately more complete. The latter effect suggests an irreversible inactivation of the enzyme rather than an equilibrium.

* This work formed part of the research programme carried out for the Ministry of Supply by an Extra-Mural Research Team under the direction of Dr M. Dixon, and was reported to the Ministry of Supply (Mackworth, 1942; Webb, 1944).

Reversibility. It was shown by Matthes (1930) that the effect of eserine on cholinesterase could be reversed completely by prolonged dialysis against water. These results have been confirmed, but on the other hand it proved impossible to obtain any reversal of the poisoning by the fluorophosphonate esters (Table 1). The enzyme solution (5 ml.) was treated with the inhibitor for 15 min. at 38°; 1 ml. was used at once for activity estimation, and the remainder dialyzed against running tap water for 24 hr. in the case of the eserine experiment, 36 hr. in the others. A control was treated in the same manner, in the absence of inhibitor, and corrections were applied for the volume change on dialysis. It is clear that the combination between the fluorophosphonate esters and the enzyme is of a much firmer nature than that between eserine and the enzyme.

Table 1. *Effect of prolonged dialysis against water on activity of cholinesterase poisoned with eserine and fluorophosphonates*

(Enzyme incubated with poison for 15 min. at 38° before dialysis.)

Inhibitor	Conc. (M)	Inhibition (%) Before dialysis	Inhibition (%) After dialysis for 24–36 hr.
Eserine	10^{-6}	100	35
Diethyl fluorophosphonate	4×10^{-9}	70	76
Diethyl fluorophosphonate	10^{-8}	90	85
Di*iso*propyl fluorophosphonate	10^{-9}	50	50

Effect of substrate concentration. In the following experiments the cholinesterase activities were measured by a continuous titration method. The digest of acetylcholine and horse serum cholinesterase (total vol. 10 ml.), containing bromothymol blue and 0·0002M-phosphate, was titrated with 0·01N-NaOH to maintain the pH at 7·4. The titrations, which were carried out in a water bath at 20°, were linear over a period of 10–15 min. The velocity was expressed as ml. 0·01N-NaOH/5 min.; under the conditions used, it was proportional to the enzyme concentration. When an inhibitor was added, this was equilibrated with the enzyme etc., for 5 min. at 20° before adding the substrate contained in a volume of 1 ml.

Under the conditions used the enzyme was markedly less sensitive to fluorophosphonate than in the manometric experiments, due probably to the higher enzyme concentration and the shorter incubation period, so that only the initial rapid inhibition took place. With an acetylcholine concentration of 0·0045M, 50% inhibition was produced by approximately 2×10^{-7}M-eserine or $3{\cdot}5 \times 10^{-8}$M-di-*iso*propyl fluorophosphonate. When the substrate concentration was varied over the range 0·0004–0·06M, the percentage inhibition by the fluorophosphonate (compared with a standard having a similar substrate concentration but no inhibitor) remained more or less constant. On the other hand, the inhibition due to eserine decreased when the acetylcholine concentration was raised. The difference in the behaviour of the two inhibitors is clearly shown when the results are examined by the method of Lineweaver & Burk (1934), which has already been applied to serum cholinesterase and eserine by Eadie (1942). The relationship between enzyme reaction velocity v and substrate concentration S is given by

$$\frac{1}{v} = \frac{1}{V} + \frac{K_p}{V}\frac{1}{S},$$

where V is the limiting velocity and K_p is the effective Michaelis constant. In the presence of a 'competitive' reversible inhibitor, V is unchanged but K_p is increased, i.e. the apparent affinity of the enzyme for its substrate is lowered. If there is no competition between inhibitor and substrate for the enzyme centres, $K_p = K_M$ but V is lowered.

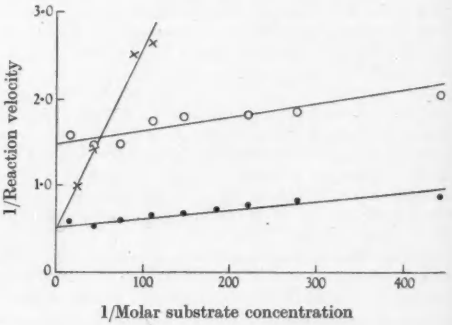


Fig. 2. Effect of substrate concentration on inhibition of horse serum cholinesterase. Enzyme activity estimated by titration with 0·01N-NaOH at pH 7·4 and 20°. ●—●, control, no inhibitor; ×—×, 2×10^{-7} M-eserine; ○—○, 5×10^{-8} M-di*iso*propyl fluorophosphonate.

Fig. 2 shows the results obtained with 2×10^{-7}M-eserine and 5×10^{-8}M-di*iso*propyl fluorophosphonate. Eserine behaves like a typical competitive

inhibitor, with V unchanged, but the fluorophosphonate gives no indication of competition with the substrate.

True and pseudo-cholinesterase. The crude serum preparation used contained both the 'true' and 'pseudo' cholinesterases of Mendel & Rudney (1943). The effect of di*iso*propyl fluorophosphonate on these components was examined separately by means of the specific substrates described by Mendel, Mundell & Rudney (1943), using the titration method described above. Fluorophosphonate (5×10^{-8} M) gave an inhibition of 57 % of the activity towards 0·0045 M-acetylcholine, 30 % of the activity towards 0·0005 M-acetyl-β-methylcholine, and 40 % of that towards 0·005 M-benzoylcholine, after incubating the enzyme with the poison for 5 min. Thus there is no appreciable difference in sensitivity of the true and pseudo-cholinesterases of horse serum to fluorophosphonates.

Assessment of inhibitory power. In order to compare the inhibitory power of the different compounds, conditions for a standard manometric test were defined. A fixed amount of enzyme was incubated at 20° with varying concentrations of the inhibitor for 15 min. before tipping in the acetylcholine. The CO_2 production in the first 10 min. was used for estimating the activity, and by comparison with a control, the percentage inhibition. Curves showing the percentage inhibition produced under the standard conditions plotted against the logarithm of the inhibitor concentration for eserine and a number of alkyl fluorophosphonates are shown in Fig. 3. They are in general sigmoid in shape, but are not all similar; in particular, those for *iso*propyl and *sec.*-butyl fluorophosphonate are considerably flatter than the others, so that in the range of inhibitions below 50 % these esters are relatively more effective than would be indicated by the pI_{50} values (see below). With different preparations of cholinesterase the absolute positions of the curves tended to vary, but they occupied the same position relative to one another. The graph for eserine agrees with that given by Ellis, Krayer & Plachte (1943). In an extended series of studies, the inhibition-concentration curves for new esters were always compared against that for di*iso*propyl fluorophosphonate, which was redetermined at the same time.

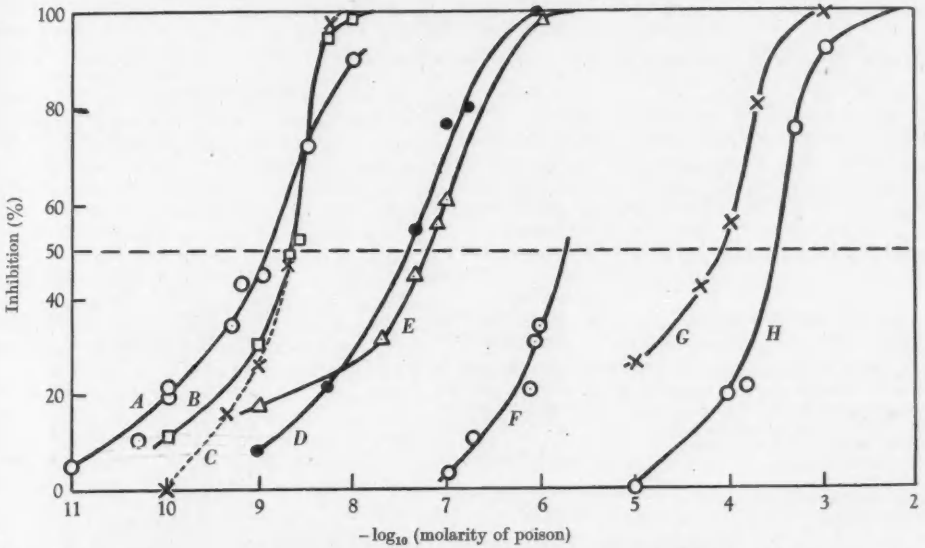


Fig. 3. Inhibition of horse serum cholinesterase by various compounds. Incubated for 15 min. at 20° before addition of 2 mg. acetylcholine chloride. A, di*iso*propyl fluorophosphonate; B, di*sec.*-butyl fluorophosphonate; C, di*iso*amyl fluorophosphonate; D, eserine; E, diphenyl fluorophosphonate; F, dithioethyl fluorophosphonate; G, dimethylaminophosphoryl fluoride; H, diethyl methylaminophosphate.

To obtain a numerical value for inhibitory power, the concentration of poison producing 50 % inhibition in the standard test was read off from the inhibition-log (concentration) curve. This value is conveniently expressed as the pI_{50}, which is the negative logarithm of the concentration producing 50 % inhibition (Mazur & Bodansky, 1944). Values for the poisons examined are given in Table 2. Di*iso*propyl fluorophosphonate was the most active

of these compounds, being thirty times more potent than eserine. The other branched-chain esters, namely *sec.*-butyl and *iso*amyl, although less active than the *iso*propyl derivative, are more active than the straight-chain *n*-propyl, ethyl and methyl esters. This suggests a special affinity of the enzyme for methyl groups, which are also present in the substrate and in eserine. Replacement of oxygen in diethyl fluorophosphonate by sulphur reduces the activity to 1/400 of the original. A slight activity is shown by methylaminophosphonate ethyl ester; unesterified fluorophosphonate is virtually inactive.

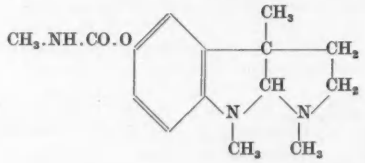
serum cholinesterase and serum lipase activities *in vivo*. Similarly, Mazur & Bodansky (1946) have observed a correlation between serum cholinesterase and poisoning by fluorophosphonates, although Hawkins & Gunter (1946) have shown that complete inhibition of the serum (pseudo) cholinesterase *in vivo* is not necessarily accompanied by symptoms of acetylcholine accumulation. The values obtained in this work for inhibitory power towards serum cholinesterase fall into the same series as those from the physiological assay on the rabbit eye, although there is not a strict quantitative agreement. Possibly

Table 2. *Inhibitory power of eserine, fluorophosphonates and related compounds on horse serum cholinesterase*

(Standard test conditions; 15 min. incubation at 20° in bicarbonate buffer, pH 7·4, in absence of substrate.)

Inhibitor	Formula	Molarity producing 50% inhibition	pI_{50} ($-\log_{10}$M)
Di*iso*propyl fluorophosphonate	$\left(\begin{matrix}CH_3\\CH_3\end{matrix}\!\!>\!CHO.\right)_2PO.F$	$1{\cdot}3\times10^{-9}$	8·9
Di*sec.*-butyl fluorophosphonate	$\left(\begin{matrix}C_2H_5\\CH_3\end{matrix}\!\!>\!CHO.\right)_2PO.F$	$2{\cdot}0\times10^{-9}$	8·7
Di*iso*amyl fluorophosphonate	$\left(\begin{matrix}C_2H_5\\CH_3\end{matrix}\!\!>\!CH.CHO.\right)_2PO.F$	$2{\cdot}0\times10^{-9}$	8·7
Di-*n*-propyl fluorophosphonate	$(CH_3.CH_2.CH_2O.)_2PO.F$	$5{\cdot}5\times10^{-9}$	8·25
Diethyl fluorophosphonate	$(C_2H_5O.)_2PO.F$	$8{\cdot}0\times10^{-9}$	8·1
Eserine	*	$4{\cdot}0\times10^{-8}$	7·4
Diphenyl fluorophosphonate	$(C_6H_5O.)_2PO.F$	$6{\cdot}3\times10^{-8}$	7·2
Dimethyl fluorophosphonate	$(CH_3O.)_2PO.F$	$1{\cdot}0\times10^{-7}$	7·0
Dithioethyl fluorophosphonate	$(C_2H_5S.)_2PO.F$	$2{\cdot}0\times10^{-6}$	5·7
Dimethylaminophosphoryl fluoride	$(CH_3)_2N.PO(OH).F$	$8{\cdot}0\times10^{-5}$	4·1
Diethyl methylaminophosphonate	$(C_2H_5O.)_2PO.NHCH_3$	$3{\cdot}0\times10^{-4}$	3·5
Trimethyl phosphate	$(CH_3)_3PO_4$	$1{\cdot}0\times10^{-3}$	3·0
Vitamin B_1	—	$1{\cdot}7\times10^{-3}$	2·8
Ammonium fluorophosphonate	$(NH_4O.)_2PO.F$	$1{\cdot}0\times10^{-2}$	2·0
Sodium fluoride	NaF	$1{\cdot}0\times10^{-2}$	2·0

* Eserine has the following structure (Polonovski & Polonovski, 1925):



DISCUSSION

The extremely high affinity for cholinesterase of the fluorophosphonates, especially those with branched-chain alkyl groups, suggests that the toxic and myotic power of these compounds is due to inhibition of cholinesterase *in vivo*. Bloch (1943) showed that the parasympatheticomimetic symptoms of poisoning by cresyl phosphate, which is also an inhibitor of cholinesterase *in vitro*, were paralleled by a fall of

better agreement would have been obtained if the compounds had been assessed on a tissue true cholinesterase.

The inhibition of cholinesterase by eserine is readily understood by comparing the structure of the alkaloid with that of acetylcholine. Eadie (1942) has shown that eserine is a competitive inhibitor of cholinesterase, and that an equilibrium is reached within a few minutes of mixing, in which two molecules of eserine combine with one molecule of enzyme. The action of other inhibitors which possess

a strongly dissociated quaternary ammonium group, such as methylene blue, miotine (Massart & Dufait, 1941), phenylpyrazolones (Zeller, 1942), and vitamin B_1, which is a quaternary thiazolinium salt (Glick & Antopol, 1939), is probably similar. The action of the alkyl fluorophosphonates, on the other hand, is probably of a different nature; it is not reversible and there is no evidence of substrate competition or of an equilibrium, the destruction being progressive for at least an hour. Nevertheless the sigmoid curves obtained when inhibition is plotted against the logarithm of inhibitor concentration simulate equilibrium curves, and the more flattened, asymmetric curves obtained for the most potent esters resemble those which Straus & Goldstein (1943) have shown are to be expected for enzyme-inhibitor equilibrium when the enzyme concentration is high relative to the enzyme-inhibitor dissociation constant (so that the degree of inhibition is dependent to some extent on the concentration of the enzyme, as well as that of the inhibitor). Probably similar curves are to be expected if the degree of inhibition is determined by the velocity of a reaction between enzyme and inhibitor, dependent on their respective concentrations. It is possible that the initial rapid inhibition is an equilibrium, but that this is followed by an irreversible destruction of the enzyme; this would be consistent with the two-phase nature of the progress curve of inhibition. In any case, the effective irreversibility of the destruction would explain the prolonged action of the fluorophosphonates *in vivo*, in contrast to the transitory effect of eserine.

Easson & Stedman (1936) and Straus & Goldstein (1943) give figures of 1490 and 450 molecules of acetylcholine/sec./enzyme centre respectively for the absolute activity of horse serum cholinesterase at 30°, based on the inhibition by eserine at varying concentrations. (These figures depend on the assumption that there is an equilibrium between one enzyme centre and one molecule of eserine. They presumably need recalculation in the light of the work of Eadie mentioned above, but are probably of the right order of magnitude.) In the manometric experiment with an amount of serum cholinesterase which produced 100 μl. CO_2/10 min. at 20°, complete inhibition was produced by about 10^{-8} M-di*iso*propyl fluorophosphonate. Calculation shows that under these conditions complete inactivation was produced in 15 min. by the presence of 1–3 molecules of fluorophosphonate/enzyme active centre, which is particularly remarkable in view of the low purity of the enzyme preparation used. Clearly the fluorophosphonate has a very specific affinity for the active centres attacked.

SUMMARY

1. The alkyl fluorophosphonates examined were found to be highly potent inhibitors of horse serum cholinesterase. The most active ester, di*iso*propyl fluorophosphonate, was 30 times as active as eserine.

2. The inhibition of cholinesterase by alkyl fluorophosphonates, unlike the action of eserine, is progressive with time. It is not affected by the substrate concentration and cannot be reversed by prolonged dialysis.

3. The most potent esters were those with short branched-chain alkyl groups. The order of inhibitory power of the various esters was roughly the same as that for toxicity and for myotic power.

4. The true and pseudo-cholinesterase components of horse serum were equally sensitive to di*iso*propyl fluorophosphonate.

The authors are grateful to Dr B. C. Saunders for the fluorophosphonates studied, to Dr E. Boyland for a gift of benzoylcholine (Roche), and to the Chief Scientist of the Ministry of Supply, for permission to publish this paper.

REFERENCES

Ammon, R. (1933). *Pflug. Arch. ges. Physiol.* **233**, 486.
Bloch, H. (1943). *Helv. chim. Acta*, **26**, 733.
Eadie, G. S. (1942). *J. biol. Chem.* **146**, 85.
Easson, L. H. & Stedman, E. (1936). *Proc. Roy. Soc.* B, **121**, 142.
Ellis, S., Krayer, O. & Plachte, F. L. (1943). *J. Pharmacol.* **79**, 309.
Glick, D. & Antopol, W. (1939). *J. Pharmacol.* **65**, 389.
Hawkins, R. D. & Gunter, J. M. (1946). *Biochem. J.* **40**, 192.
Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
McCombie, H. & Saunders, B. C. (1946). *Nature, Lond.*, **157**, 286.
Mackworth, J. F. (1942). Report by Dixon to the Ministry of Supply, no. 13.*
Massart, L. & Dufait, R. (1941). *Enzymologia*, **9**, 364.
Matthes, K. (1930). *J. Physiol.* **70**, 338.
Mazur, A. & Bodansky, O. (1944). By communication.*
Mazur, A. & Bodansky, O. (1946). *J. biol. Chem.* **163**, 261.
Mendel, B., Mundell, D. B. & Rudney, H. (1943). *Biochem. J.* **37**, 473.
Mendel, B. & Rudney, H. (1943). *Biochem. J.* **37**, 59.
Polonovski, M. & Polonovski, M. (1925). *Bull. Soc. chim. Fr.* **37**, 744.
Stedman, E. & Stedman, E. (1935). *Biochem. J.* **29**, 2563.
Straus, O. H. & Goldstein, A. (1943). *J. gen. Physiol.* **26**, 559.
Webb, E. C. (1944). Report by Dixon to the Ministry of Supply, no. 27.*
Zeller, E. A. (1942). *Helv. chim. Acta*, **25**, 1099.

* Information available on application to Ministry of Supply, London.

The Action of Alkyl Fluorophosphonates on Esterases and other Enzymes

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Work carried out during the war showed that alkyl fluorophosphonates, especially those with short branched-chain alkyl groups, have a very powerful inhibitory action on serum cholinesterase *in vitro* (Mackworth & Webb, 1948), and it was at first supposed that this action might be specific for cholinesterase. To test this hypothesis, the action of a potent alkyl fluorophosphonate was studied on a number of enzymes *in vitro*, and the results are reported in this paper. They show that esterases other than serum cholinesterase are sensitive to fluorophosphonate.

EXPERIMENTAL AND RESULTS

Tissue cholinesterase. The cholinesterase activity of a rabbit brain preparation, obtained by grinding the brain in bicarbonate Ringer solution and straining through muslin, was estimated in Barcroft manometers in the presence of 8 mg. acetylcholine. Under the conditions of treatment reported in the previous paper for serum cholinesterase, the brain enzyme was 50% inhibited by $6{\cdot}7 \times 10^{-8}$ M-ethyl fluorophosphonate, or by 5×10^{-7} M-eserine. Thus the inhibitory activity of diethyl fluorophosphonate against rabbit brain cholinesterase ($pI_{50} = 7{\cdot}2$) is about one eighth of its activity against horse serum cholinesterase ($pI_{50} = 8{\cdot}1$) but the relative activities of the two inhibitors on each enzyme remains the same.

Liver esterase. Simple esterase was estimated, with ethyl butyrate as substrate, by the continuous titration method of Harrer & King (1941). The titration mixture (10 ml.) contained M/450 ethyl butyrate, M/5000 phosphate and 0·003% bromothymol blue, and was maintained at pH 7·4 by titration with 0·01 N-NaOH at 20°. The enzyme had been partially purified from an aqueous extract of acetone-dried ox liver by fractional precipitation with ammonium sulphate and fractional adsorption on calcium phosphate gel. It had no detectable action on acetylcholine (cholinesterase activity/simple esterase activity $< 1/1500$).

Table 1 shows the inhibition of the enzyme by fluorophosphonate, eserine, and fluoride. To prevent reversal of the inhibition on dilution, the enzyme was incubated with the poison in a volume of 2 ml. for 15 min. at 20°, and then the reaction was started by adding 8 ml. of substrate, phosphate, etc., containing the same concentration of poison as the enzyme solution. It will be seen that liver esterase is strongly inhibited by fluorophosphonate but is relatively insensitive to eserine and fluoride.

Table 1. *Inhibition of liver esterase*

(Ethyl butyrate as substrate. Enzyme incubated with poison for 15 min. at 20° before estimation.)

Inhibitor	Conc. (M)	Inhibition (%)
Di*iso*propyl fluorophosphonate	10^{-6}	91
	5×10^{-7}	77
	3×10^{-7}	32
	10^{-7}	<10
Eserine	10^{-2}	65
	10^{-3}	25
NaF	10^{-2}	>85
	2×10^{-3}	73
	10^{-3}	48
	10^{-4}	7

Milk lipase. Human milk contains a powerful lipase, which can easily be estimated by a continuous titration method (Hottinger & Bloch, 1943). The most convenient substrate was a 1·5% emulsion of tributyrin in 1% sodium glycocholate (British Drug Houses Ltd,), neutralized to pH 7·5. A sample of human milk (4 weeks *post partum*) was skimmed by centrifuging and used directly as the enzyme; 0·06 ml. was sufficient for an activity estimation with tributyrin. The enzyme, together with 0·5 ml. 0·02 M-phosphate and 0·3 ml. 0·1% indicator (bromothymol blue or cresol red) was diluted to 8 ml., and the reaction started by addition of 2 ml. of the tributyrin emulsion. The mixture, contained in a boiling tube, was shaken gently and continuously in a water bath at 20°, and 0·01 N-NaOH was run in from a micro-burette to maintain the pH constant. The titration was completely linear over a period of 10–15 min. for a total addition of 1–2 ml. NaOH, the velocity being directly proportional to the amount of enzyme taken. Control titrations, (*a*) without enzyme and (*b*) without substrate, were zero. The optimal pH was found to be 8·0 (± 0·2), and the experiments with poisons were carried out at pH 8 with cresol red as indicator, after a preliminary 15 min. incubation of enzyme and poison

at pH 7·4. A few experiments were carried out with other substrates, and the activities obtained are shown in Table 2.

Table 2. *Hydrolytic activity of skimmed human milk on various substrates*

(At pH 8, 20°, in presence of 0·2% glycocholate. 1 unit = the amount of enzyme which produces free acid equivalent to 1 ml. 0·01 N-NaOH in 5 min.)

Substrate	Activity (units/ml. milk)
Tributyrin	17·5
Olive oil	0·25
Ethyl butyrate	0·28
Acetylcholine	⩽0·004

The tributyrinase activity was strongly inhibited by fluorophosphonate, considerably less so by eserine, and was insensitive to fluoride. The inhibition by eserine increased continuously, even after addition of substrate, to a maximum at 2 hr. (Table 3). Similar results were obtained with olive oil as substrate.

Kidney phosphatase. The enzyme was prepared from rabbit kidney by the method of Delory & King (1944), and was estimated by the method of Bodansky (1933), using sodium β-glycerophosphate as substrate. The acid phosphatase activity was only moderately sensitive to fluorophosphonate, the alkaline phosphatase still less so (Table 4). According to Belfanti, Contardi & Ercoli (1935) acid phosphatase is sensitive to fluoride, while alkaline phosphatase is not.

Yeast pyrophosphatase. The purified yeast pyrophosphatase of Bailey & Webb (1944) was 48% inhibited by 10^{-3} M-di*iso*propyl fluorophosphonate, and unaffected by eserine. However, it was far more sensitive to free fluoride (50% inhibition by 2×10^{-5} M-NaF), and the result obtained with fluorophosphonate might be due to about 2% of free fluoride present as impurity in the preparation.

Enzymes insensitive to fluorophosphonate. Diethyl and dimethyl fluorophosphonates were found to be without effect on the following enzymes, when tested in concentrations up to 0·002 M: choline dehydrogenase, cytochrome oxidase, catalase, peroxidase, succinic dehydrogenase, lactic dehydrogenase (of muscle), alcohol dehydrogenase (of liver), yeast hexokinase, carboxylase, amylase, diaphorase, carbonic anhydrase, and the glycolytic system of muscle.

Table 3. *Inhibition of human milk lipase*

(At pH 8, 20°; in 0·2% glycocholate, 0·001 M-phosphate. Preliminary incubation at pH 7·4.)

Substrate	Inhibitor	Concentration (M)	Time of incubation without substrate (min.)	Inhibition (%)
Tributyrin	Di*iso*propyl fluorophosphonate	10^{-4}	15	100
		10^{-5}	15	66
		5×10^{-6}	15	50
		2×10^{-6}	15	32
		10^{-6}	15	16
Tributyrin	Eserine	10^{-3}	15	35–60
		10^{-3}	120	>97
		3×10^{-4}	120	86
		10^{-4}	120	50
Tributyrin	NaF	10^{-1}	15	40
		10^{-2}	15	0
Olive oil	Di*iso*propyl fluorophosphonate	10^{-5}	15	77
Olive oil	Eserine	10^{-3}	15	60–95

Table 4. *Inhibition of kidney phosphatases*

(38°, 0·02 M-glycerophosphate. Acid phosphatase estimated in 0·1 M-acetate, pH 5·5. Alkaline phosphatase estimated in 0·08 M-veronal-acetate buffer pH 9, containing 0·005 M-$MgCl_2$. Enzyme and poison incubated for 15 min. before addition of substrate.)

Inhibitor	Concentration (M)	pH	Inhibition (%)
Di*iso*propyl fluorophosphonate	10^{-3}	5·5	60
	3×10^{-4}	5·5	5
	10^{-4}	5·5	0
Eserine	10^{-3}	5·5	18
Di*iso*propyl fluorophosphonate	10^{-3}	9·0	12
Eserine	10^{-3}	9·0	7

Table 5. *Sensitivity of various esterases to fluorophosphonate ester, eserine and fluoride*

($pI_{50} = -\log_{10}$ (molarity producing 50% inhibition).)

Enzyme	pI_{50}		
	Di*iso*propyl fluorophosphonate	Eserine	NaF
Serum cholinesterase	8·9*	7·4*	2·0*
	7·45	6·7	—
Liver esterase	6·4	*c.* 2·5	*c.* 3
Human milk lipase	5·3	*c.* 3 (15 min. incubation)	<1
	—	*c.* 4 (2 hr. incubation)	—
Kidney acid phosphatase	*c.* 3	<3	(+)
Kidney alkaline phosphatase	≪3	≪3	(−)
Yeast pyrophosphatase	3 (?)	0	4·7

* These values were calculated from manometric results; all the others were obtained by a titration method.

DISCUSSION

It is clear that the fluorophosphonates are not, as was at first supposed, specific inhibitors of cholinesterase. They poison a range of esterases, some of which have no activity at all towards acetylcholine. In this they resemble tri-*o*-cresyl phosphate and tri-*o*-chlorophenyl phosphate, which have been shown to inhibit serum and tissue cholinesterase, serum lipase, and human milk lipase, but not pancreatic lipase (Bloch, 1943; Hottinger & Bloch, 1943). Bloch has drawn attention to the fact that the enzymes which are inhibited by cresyl phosphate are also sensitive to eserine. However, Table 5 shows that there is no quantitative relationship between the sensitivity of enzymes to fluorophosphonate and to eserine, or between the sensitivity to fluorophosphonate and to free fluoride.

Mackworth & Webb (1948) in the preceding paper gave reasons for believing that the action of fluorophosphonates on serum cholinesterase is of quite a different nature from the action of eserine on the enzyme. The distinction is emphasized by the differing specificity of inhibition reported above; in particular, by the powerful action of fluorophosphonate on simple esterase and milk lipase, which are comparatively insensitive to eserine.

SUMMARY

1. Brain cholinesterase was inhibited by an alkyl fluorophosphonate, but not as strongly as serum cholinesterase.

2. Liver esterase, human milk lipase and kidney phosphatase were also sensitive to di*iso*propyl fluorophosphonate, though not to the same extent as cholinesterase. These enzymes were also inhibited by eserine, but there was no correlation between the sensitivity towards fluorophosphonate and towards eserine or sodium fluoride. Liver esterase in particular is highly sensitive to fluorophosphonate but relatively insensitive to eserine. The specificity of inhibition by fluorophosphonates is similar to that reported for tri-*o*-cresyl phosphate and tri-*o*-chlorophenyl phosphate (Bloch, 1943).

3. Alkyl fluorophosphonates had no effect on a number of other enzymes tested, with the possible exception of yeast pyrophosphatase.

The author is grateful to Dr B. C. Saunders for the methyl, ethyl and *iso*propyl fluorophosphonates used.

REFERENCES

Bailey, K. & Webb, E. C. (1944). *Biochem. J.* **38**, 394.
Belfanti, S., Contardi, A. & Ercoli, A. (1935). *Biochem. J.* **29**, 517.
Bloch, H. (1943). *Helv. chim. Acta*, **26**, 733.
Bodansky, A. (1933). *J. biol. Chem.* **101**, 93.
Delory, G. E. & King, E. J. (1944). *Biochem. J.* **38**, 50.
Harrer, C. J. & King, C. G. (1941). *J. biol. Chem.* **138**, 111.
Hottinger, A. & Bloch, H. (1943). *Helv. chim. Acta*, **26**, 142.
Mackworth, J. F. & Webb, E. C. (1948). *Biochem. J.* **42**, 91.

The Synthesis of Some Dipeptides Related to Gramicidin S

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This paper describes the synthesis of five dipeptides required for study of the structure of gramicidin S (Consden, Gordon, Martin & Synge, 1947). For all the syntheses carbobenzoxy derivatives were employed according to Bergmann's general procedure; coupling was effected in each case through the acid chloride. Fair yields were obtained at all stages, and no special difficulties were encountered. This appears to be the first time that peptides of valine or of ornithine have been synthesized by the carbobenzoxy procedure. For synthesizing α-(L-valyl)-L-ornithine it was necessary to block the δ-amino group of ornithine with the carbobenzoxy radical. This was done by both the procedures that have been used for homologous preparations with lysine, i.e. (1) through the *NN'*-dicarbobenzoxy derivative, its acid chloride and the cyclic α-*N*-carboxy anhydride (Bergmann, Zervas & Ross, 1935); (2) through the copper complex of the free amino-acid (Neuberger & Sanger, 1943; cf. Kurtz, 1938). Sanger (1946) has already employed the latter procedure for the preparation of δ-*N*-2:4-dinitrophenylornithine. Both procedures gave similar yields, but the copper method involved somewhat simpler manipulations. The fact that the monocarbobenzoxyornithine obtained reacted with ninhydrin to yield one molecule of CO_2 provided further evidence that it was the δ-carbobenzoxy derivative. Van Slyke, Dillon, MacFadyen & Hamilton (1941) have demonstrated that ε-carbobenzoxylysine yields one molecule of CO_2 with ninhydrin.

The advantages of using dilute anhydrous HCl in cold methanol for esterifying proteins and other compounds somewhat readily split by acid, such as *N*-acylamino-acids, have been demonstrated by Fraenkel-Conrat & Olcott (1945). The present work has shown that the method is applicable to carbobenzoxy derivatives of amino-acids (δ-carbobenzoxyornithine, carbobenzoxyvaline) and to free amino-acids (valine, leucine).

The purity of the resulting dipeptides has been checked in every case by paper-strip chromatograms with a suitable solvent system (cf. Consden, Gordon & Martin, 1944). In some preliminary experiments the products, when tested in this way, showed, in addition to the desired compound, a complex array of contaminants that coloured with ninhydrin. Such results occurred in the syntheses of valylornithine and leucylphenylalanine, if the coupling was effected with acid chloride that had been purified in the usual way by washing with ligroin; in these cases the treatment appears inadequate to remove phosphorus compounds that may have deleterious effects. It was found that washing of the acid chlorides with water, as in the preparation of carbobenzoxyleucyl chloride by Bergmann, Zervas & Fruton (1936), gave much purer products. Good results were also obtained in this way in the synthesis of prolylvaline. It appears, as pointed out to me by Dr C. R. Harington, that these acid chlorides are much less sensitive to moisture than would appear from the literature. It was also found that the coupling reactions, in the syntheses of valylornithine and leucylphenylalanine, proceeded much more rapidly, giving better yields, in organic solvents shaken with saturated aqueous $KHCO_3$, than in dry mixtures of organic solvents. This procedure also effects a valuable economy in the amount of ester that has to be used.

A point worth re-emphasizing is the importance of the general practice of employing amino-acid esters rather than free amino-acids in the coupling. In the present work, in view of the reported instability of proline ester to moisture (Abderhalden & Sickel, 1926), and following the procedure of Bergmann, Zervas, Schleich & Leinert (1932) for making peptides of proline, free proline was employed for the synthesis of phenylalanylproline. The separation of the desired carbobenzoxydipeptide from carbobenzoxyamido-acid resulting from decomposition of the acid chloride with water is then difficult. Furthermore, the free carboxyl groups of free amino-acid and/or carbobenzoxydipeptide may react with unchanged acid chloride to form mixed anhydrides, which in turn can react with more amino-acid, and so on, to form a complicated mixture of peptide derivatives. Both these difficulties are avoided by adhering to the use of the esters for coupling, and, in view of the impurity associated with the product of the phenylalanylproline synthesis described here, it would be worth searching for a derivative of proline for such syntheses in which the carboxyl group is blocked by a suitable radical. A similar formation of mixed anhydrides of acetic acid with acetamido-acids may explain the difficulty of quantitatively converting the mixture of amino-acids in a protein hydrolysate to acetamido-acids for chromatographic analyses (Gordon, Martin & Synge, 1943;

Tristram, 1946). Some of the 'artefacts' observed in such analyses, on this view, might be acetyl-peptides.

EXPERIMENTAL

General technical notes

Unless otherwise stated, compounds were recrystallized before analysis until the recorded m.p. and/or optical rotation (usually both) were substantially constant. M.p.'s are given uncorrected. Optical rotations were determined in a 0·5 dm. tube. Evaporations *in vacuo* were conducted below 40° unless otherwise stated.

One-dimensional paper partition chromatograms were done according to Consden *et al.* (1944). The R_F values observed for the dipeptides are given by Consden *et al.* (1947).

Nitrogen determinations were made by a micro-Kjeldahl procedure. For the estimation of the acid equivalent weight (AEW), the sample was titrated in aqueous ethanol with 0·01 N-$Ba(OH)_2$, employing phenolphthalein as internal indicator. The titration mixture was subsequently used for N determination. C, H and Cl (Carius) determinations were by Dr G. Weiler, Oxford.

Catalytic reduction of carbobenzoxy compounds was effected in the usual way; 50–250 mg. of the carbobenzoxy compound were dissolved in 1 ml. glacial acetic acid, 10 ml. methanol and 1–2 ml. water, and hydrogenated at atmospheric pressure and room temperature in the presence of 0·1–0·2 g. active Pd (Willstätter & Waldschmidt-Leitz, 1921). In the case of the carbobenzoxy derivatives of the ornithine peptides, 2–3 mol. equiv. of HCl were added in the water, and in the preparation of ornithylleucine 10 ml. acetic acid and no methanol were arbitrarily employed in the solvent mixture. In every case CO_2 evolution was complete after 0·5–1 hr.

Starting materials. The amino-acids employed for the present syntheses were prepared in this laboratory, and tested for the absence of expected contaminant amino-acids with suitable solvents on paper partition chromatograms. Stereochemical purity was checked in each case by determinations of optical rotation of the amino-acid or its formyl derivative.

The racemic synthetic amino-acids were resolved through their formyl derivatives by the methods of Fischer (1906). After characterization, the formyl derivatives were hydrolyzed with hydrochloric acid (the strength of acid and duration of refluxing being as given by Fischer), and the hydrolysate was evaporated to dryness *in vacuo* up to 100° several times with water. The resulting amino-acid hydrochlorides were used directly for the syntheses.

L-*Ornithine monohydrochloride.* L-Arginine monohydrochloride was prepared from a HCl hydrolysate of gelatin (Cox, 1928). From this, L-ornithine monohydrochloride ($[\alpha]_D^{18°}$ +10·8° (water, $c=5$)) was prepared by the arginase method of Hunter (1939).

L-*Proline.* The above gelatin hydrolysate, freed from arginine by flavianic acid, was subjected to the rhodanilate procedure of Bergmann (1935) for the isolation of L-proline. The product, dried to constant weight at 60° over P_2O_5 *in vacuo* had $[\alpha]_D^{19°}$ −81·9° (water, $c=7·0$).

L-*Valine.* *iso*Propanol was converted to *iso*propyl bromide (Norris, 1907) and this, by malonic ester synthesis (Marvel & du Vigneaud, 1943) to α-bromo*iso*valeric acid, which was aminated (Marvel, 1940) to DL-valine. This was resolved (Fischer, 1906) yielding formyl-L-valine, m.p. 151–152°, $[\alpha]_D^{18°}$ −16·6° (water, $c=5·0$).

L-*Leucine.* *iso*Butyl bromide, by malonic ester synthesis (Marvel & du Vigneaud, 1943) yielded α-bromo*iso*caproic acid which was aminated (Marvel, 1941) to DL-leucine. This was resolved (Fischer & Warburg, 1905) yielding formyl-L-leucine, m.p. 137–140°, $[\alpha]_D^{18°}$ −18·6 (ethanol, $c=8·7$).

D-*Phenylalanine.* DL-Phenylalanine was prepared from hippuric acid and benzaldehyde (Gillespie & Snyder, 1943). It was resolved (Fischer & Schoeller, 1907) to yield formyl-D-phenylalanine, m.p. 164–166°, $[\alpha]_D^{18°}$ −74·5° (ethanol, $c=3·3$).

Substitution products of ornithine used as intermediates

(*a*) δ-*Carbobenzoxy-L-ornithine methyl ester through the Cu derivative of ornithine.* A solution of L-ornithine monohydrochloride (5 g.) in 50 ml. water was saturated with $CuCO_3$ at 100°, cooled and filtered. The resulting deep-blue solution was treated with 2 N-NaOH and benzyl chloroformate, added alternately in 6 equal portions over a period of 20 min. at 0°, whilst the flask was vigorously shaken. In all, 30 ml. 2 N-NaOH and 6 ml. benzyl chloroformate were added. The mixture was then shaken mechanically at room temperature for 30 min. The pale-blue precipitate was then filtered off and thoroughly washed on the filter, first with water, and then with ethanol. The air-dry precipitate was suspended in 150 ml. water, and treated with H_2S overnight in a closed vessel. Water (550 ml.) was then added, the mixture was boiled for a few minutes and rapidly filtered from CuS through a pre-heated filter. The CuS was further washed with 300 ml. of boiling water. On cooling the combined filtrate and washings, δ-*carbobenzoxy-L-ornithine* (3 g.) crystallized out in needles. The mother liquors yielded on concentration *in vacuo* only 0·3 g. of inferior material which was not utilized. $[\alpha]_D^{16°}$ +17° (in 1 : 1 v/v aqueous acetone in the presence of 2 mol. HCl, $c=2·9$). Found: C, 58·8; H, 7·3; N, 10·5. $C_{13}H_{18}O_4N_2$ requires C, 58·6; H, 6·8; N, 10·5%. A ninhydrin-CO_2 determination (Van Slyke *et al.* 1941) at a total volume of 1·25 ml., pH 2·5, with 50 mg. ninhydrin gave carboxyl N, 5·2%. (Theory requires 5·3%.)

2 g. δ-Carbobenzoxy-L-ornithine were dissolved in 100 ml. anhydrous methanol, N in respect of HCl, and kept for 24 hr. at room temperature. The mixture was evaporated to dryness *in vacuo*. After two further such treatments with methanolic HCl, the residue crystallized rapidly and completely. It was recrystallized from hot acetone, ether being added to increase the completeness of recovery. The yield of δ-*carbobenzoxy-L-ornithine methyl ester hydrochloride* was 2·35 g. For characterization see (*c*) below.

(*b*) α*δ-Dicarbobenzoxy-L-ornithyl chloride, and* δ-*carbobenzoxy-L-ornithine methyl ester therefrom.* 1·25 g. L-ornithine monohydrochloride were dissolved in 8 ml. 2 N-NaOH and to the mixture, cooled to 0°, were added alternately, with shaking, four 4·5 ml. portions of 2 N-NaOH and four 0·9 ml. portions of carbobenzoxy chloride. After some minutes the mixture was acidified with 10 N-HCl (5·5 ml.). The oil which was precipitated was taken up in 25 ml. ether, and the ether extract was counter-extracted with four successive 50 ml. portions of 7% (w/v) $KHCO_3$ solution. The combined

$KHCO_3$ extracts were acidified with 25 ml. 10N-HCl, and extracted with ether containing some ethyl acetate. The organic solvent layer was after drying evaporated to dryness *in vacuo*. The syrupy residue (2·7 g.) was crystallized from ether-light petroleum. The product was filtered off, washed with light petroleum and dried (2·47 g.). *αδ-Dicarbobenzoxy-L-ornithine* was recrystallized for analysis from ether-light petroleum, and had m.p. 112–114°, $[\alpha]_D^{20°}$ −4° (ethanol, $c=3$). Found: C, 62·6; H, 6·1; N, 6·8; AEW, 376. $C_{21}H_{24}O_6N_2$ requires C, 63·0; H, 6·0; N, 7·0%; AEW, 400.

0·64 g. αδ-Dicarbobenzoxy-L-ornithine and 0·35 g. PCl_5 were pulverized separately and then shaken together in dry ether (12 ml.) in a glass-stoppered vessel at 5–10°. Before all of the reactants had dissolved, a fluffy crystalline precipitate of *αδ-dicarbobenzoxy-L-ornithyl chloride* had begun to form. When no further obvious reaction was occurring, the mixture was cooled to 0° for 1 hr., with intermittent shaking. Dry ligroin (8 ml.) was then added, and the mixture was kept at 0° for a further hour. The crystals were then filtered off and dried. Yield, 0·63 g. of material having m.p. (sealed tube) 54–56°. The product was not analyzed, and was used immediately in the synthesis of ornithyl-leucine.

For the preparation of δ-carbobenzoxyornithine derivatives, as in the preparation of ε-carbobenzoxylysine derivatives (Bergmann *et al.* 1935), it was not necessary to isolate the acid chloride. A preparation was made as above from 0·84 g. αδ-dicarbobenzoxy-L-ornithine. Instead, however, of adding ligroin to the reaction mixture, some dry ethyl acetate was added, whereupon the crystals dissolved. The mixture was evaporated to dryness at room temperature, yielding a crystalline residue. On heating *in vacuo* to 60° the crystals melted, and the resulting syrup gassed freely. It was dissolved in dry ethyl acetate and evaporated to dryness *in vacuo* twice (odour of benzyl chloride) and further heated for 1 hr. at 40–50°. The syrupy residue was then dissolved in ethyl acetate and brought to crystallization by addition of ether and light petroleum. After one recrystallization, 0·45 g. of product resulted. *δ-Carbobenzoxy-L-ornithine-α-N-carboxy anhydride* had m.p. 86–88°, $[\alpha]_D^{19°}$ −18° (ethyl acetate, $c=4$). Found: C, 57·2; H, 6·0; N, 9·3. $C_{14}H_{16}O_5N_2$ requires C, 57·5; H, 5·5; N, 9·6%. The compound appeared more stable than the corresponding lysine derivative (Bergmann *et al.* 1935), since after storage in a desiccator for 6 months the appearance and m.p. were unchanged.

0·4 g. of the anhydride was dissolved in 15 ml. anhydrous methanol, N in respect of HCl. Immediate evolution of gas was noticed. The mixture was warmed to 50°, and then kept overnight at room temperature. On evaporation *in vacuo* the product crystallized. Recrystallization from acetone-ether yielded 0·34 g. of *δ-carbobenzoxy-L-ornithine methyl ester hydrochloride*. The properties and analytical data are given below.

(*c*) *Comparison of δ-carbobenzoxy-L-ornithine methyl ester hydrochloride prepared by the two methods.* A comparison of specimens obtained by procedures (*a*) and (*b*) above gave the following results. M.p. (*a*) 132–134°; (*b*) 132–134°; mixture, 132–134°. $[\alpha]_D^{19}$ (*a*) +14°, +15°, (*b*) +13° (methanol, $c=4$). Found: C, (*a*) 52·6, (*b*) 52·9; H, (*a*) 6·6, (*b*) 6·5; N, (*a*) 8·8, (*b*) 8·5; Cl, (*a*) 13·1, (*b*) 11·1%. $C_{14}H_{20}O_4N_2$·HCl requires C, 53·1; H, 6·6; N, 8·8; Cl, 11·2%.

α-(L-Valyl)-L-ornithine

The evaporated hydrolysate from 1·9 g. formyl-L-valine (see above) was dissolved in 2N-NaOH (16 ml.) and carbobenzoxylated as above using in all 8·25 ml. 2N NaOH and 2·6 ml. benzyl chloroformate. The mixture was shaken at room temperature for a further 20 min. and acidified with 4 ml. 10N-HCl. The oil which precipitated was extracted into about 20 ml. ether, and the ether extract was counter-extracted with five successive 16 ml. portions of 7% (w/v) $KHCO_3$ solution. The combined $KHCO_3$ extracts were acidified with 10N-HCl, and extracted with an equal volume of ether. The ether extract was evaporated *in vacuo* (50°) to a clear syrup (2·6 g.). After some weeks of storage in a desiccator, the syrup crystallized, and after recrystallization from ether-light petroleum, *carbobenzoxy-L-valine* was found to have m.p. 64–65°, $[\alpha]_D^{20°}$ +4° (ethanol, $c=2·9$). Found: C, 62·6; H, 7·0; N, 5·5; AEW, 251. $C_{13}H_{17}O_4N$ requires C, 62·2; H, 6·8; N, 5·6%; AEW, 251. The methyl ester was prepared from carbobenzoxy-L-valine by treatment with methanolic HCl. 0·38 g. of syrupy carbobenzoxy-L-valine was dissolved in 15 ml. methanol N in respect of HCl, and kept at room temperature for 24 hr. At the end of this time the mixture was evaporated to dryness *in vacuo*: the whole treatment was repeated and the residue was dissolved in ether and water. The ether layer was separated, washed with $KHCO_3$ solution, then with water, and evaporated to dryness *in vacuo*. The product (0·39 g.) crystallized readily, and was purified by recrystallization from ether-light petroleum. *Carbobenzoxy-L-valine methyl ester* was found to have m.p. 56–57°, $[\alpha]_D^{17°}$ −16° (ethanol, $c=3·1$). Found: C, 63·6; H, 7·2; N, 5·1. $C_{14}H_{19}O_4N$ requires C, 63·4; H, 7·2; N, 5·3%.

Carbobenzoxy-L-valyl chloride was frequently obtained crystalline on evaporating *in vacuo* the product of reaction of equimolecular quantities of carbobenzoxy-L-valine and PCl_5 in dry ether. It was not, however, characterized, and was best used for the coupling with δ-carbobenzoxy-L-ornithine methyl ester without isolation, as follows. 0·77 g. carbobenzoxy-L-valine was dissolved in 8 ml. ether (dried over Na) and 0·75 g. powdered PCl_5 was added. The temperature was maintained initially at 0°, but as solution of the PCl_5 occurred rather slowly, the mixture was allowed to approach room temperature. When the PCl_5 was completely or nearly dissolved, the mixture was diluted with a further 8 ml. ether and quickly washed twice with equal volumes of ice water. The ether layer was quickly treated with Na_2SO_4 and the solution was then mixed with 30 ml. of a solution of δ-carbobenzoxy-L-ornithine methyl ester in ethyl acetate. (The ester was liberated from 1·25 g. of its hydrochloride with K_2CO_3 in the usual way into ether; after evaporation *in vacuo* the syrupy ester was dissolved in ethyl acetate.) To the reaction mixture were added 7 ml. of a saturated solution of $KHCO_3$. The mixture was kept at room temperature and shaken at intervals over a period of 2 hr. Water was then added, and the organic solvent layer was washed successively with water, N-HCl, water, 7% (w/v) $KHCO_3$, and water, partially dried with Na_2SO_4, and evaporated to dryness *in vacuo*, giving 0·55 g. of a colourless, crystalline residue smelling of benzyl chloride. Recrystallization from chloroform-light petroleum yielded 0·41 g. of material satisfactory for further work. *α-(Carbobenzoxy-L-valyl)-δ-carbobenzoxy-L-ornithine methyl ester* had

m.p. 155–156°, $[\alpha]_D^{18°} \pm 0°$ (B.P. chloroform, $c = 2{\cdot}5$). Found: C, 62·7; H, 6·9; N, 8·1. $C_{27}H_{35}O_7N_3$ requires C, 63·2; H, 6·8; N, 8·2%.

0·20 g. of this compound was saponified by dissolving in 3 ml. dioxan and adding 2 ml. N-NaOH. Crystals precipitated, but these dissolved on gently shaking for 10 min. After a further 30 min. the mixture was diluted with water to about 50 ml. On acidification with 0·4 ml. 10 N-HCl an oil was precipitated, which crystallized rapidly. Yield, 0·19 g. *α-(Carbobenzoxy-L-valyl)-δ-carbobenzoxy-L-ornithine* was recrystallized from 20 parts of methanol by gradual addition of water and had m.p. 193–195°, $[\alpha]_D^{20°} - 11°$ (glacial acetic acid, $c = 0{\cdot}9$). Found: C, 62·5; H, 6·6; N, 8·3; AEW, 504. $C_{26}H_{33}O_7N_3$ requires C, 62·5; H, 6·6; N, 8·4%; AEW, 499.

0·15 g. was hydrogenated as described above. The syrupy residue was kept in a high vacuum in a desiccator over soda lime, dissolved in aqueous ethanol, and neutralized to bromothymol blue with NH_3. The mixture was evaporated *in vacuo* to dryness and treated with ethanol. Crystallization resulted, yielding *α-(L-valyl)-L-ornithine monohydrochloride (hydrated)* (0·08 g.) which was recrystallized from small volumes of water by addition of much ethanol. The optical rotation before and after recrystallization was respectively $[\alpha]_D^{19°} + 18°$ and $+19°$ (water, $c = 2$). The recrystallized material showed two very faint contaminants on a paper-strip chromatogram developed with *s*-collidine, one moving faster, and the other slower than the principal zone. The product was deliquescent, and was stored in a sealed tube. The rotation determination and analyses are referred to material dried in a vacuum desiccator over H_2SO_4-soda lime. Found: C, 43·8; H, 8·1; N, 15·0; amino N (Van Slyke, 0·5 hr. reaction time), 10·8; Cl, 13·9. $C_{16}H_{31}O_5N_3.HCl.\frac{1}{2}H_2O$ requires C, 43·4; H, 8·3; N, 15·2; amino N, 10·1; Cl, 12·8%.

L-*Ornithyl*-L-*leucine*

L-Leucine methyl ester hydrochloride (Abderhalden & Spinner, 1919; cf. Smith & Brown, 1941) was prepared in nearly theoretical yield from L-leucine by the same method as used for the preparation of δ-carbobenzoxy-L-ornithine methyl ester. 0·58 g. of the ester hydrochloride was decomposed with K_2CO_3 in the usual way. To the dry ethereal solution of the free ester (20 ml.) at 0° was added a solution of 0·63 g. αδ-dicarbobenzoxy-L-ornithyl chloride in 5 ml. ethyl acetate. Immediate separation of needles of leucine methyl ester hydrochloride resulted. The mixture was stored overnight at 0°; the crystals were then filtered off, and the filtrate was washed successively with water, N-HCl, 7% (w/v) $KHCO_3$ solution (twice) and water, and evaporated to dryness *in vacuo*. The syrupy residue gave crystals from chloroform-light petroleum (0·66 g.). *αδ-Dicarbobenzoxy-L-ornithyl-L-leucine methyl ester* was recrystallized from ethyl acetate-ether-light petroleum, and had m.p. 83–84°, $[\alpha]_D^{20°} - 18°$ (ethanol, $c = 2{\cdot}8$). Found: C, 63·7; H, 7·1; N, 7·9. $C_{28}H_{37}O_7N_3$ requires C, 63·8; H, 7·0; N, 8·0%. 0·57 g. of this compound was dissolved in 2·1 ml. dioxan and saponified by addition of 2·1 ml. N-NaOH. The mixture was kept for 0·5 hr. after becoming homogeneous and was then diluted with water to 35 ml. On acidifying with 10 N-HCl oil was precipitated, which soon crystallized, yielding 0·535 g. of crystalline material, having m.p. 60–61°, and suitable for the next step. The material was *αδ-dicarbobenzoxy-L-ornithyl-L-leucine* crystallizing, according to the analytical data, with 0·5 mol. dioxan, not removable by drying *in vacuo* at temperatures below the m.p. Recrystallization from methanol-H_2O yielded hydrated material from which the water could likewise not be removed at temperatures below the m.p. This material, dried in a desiccator at room temperature, had m.p. 63–64°, $[\alpha]_D^{17°} - 14°$ (methanol, $c = 4{\cdot}2$). Found: C, 59·4; H, 6·8; N, 7·6; AEW, 527. $C_{27}H_{35}O_7N_3.2H_2O$ requires C, 59·0; H, 7·1; N, 7·7%; AEW, 549.

0·5 g. (with dioxan of crystallization) was hydrogenated as described above. The product, a colourless gum, was dissolved in water (slight turbidity) and the solution was neutralized to bromothymol blue by addition of ammonia. The mixture was again evaporated to dryness *in vacuo*, and on treatment with ethanol yielded 0·125 g. of crystals, which were recrystallized from very concentrated aqueous solution by addition of ethanol. The analytical data suggest that the product was *L-ornithyl-L-leucine monohydrochloride* incorporating half a molecule of water of crystallization and somewhat contaminated with the dihydrochloride. It was not deliquescent, and showed on a paper chromatogram developed with *s*-collidine a very faint trace of contaminant material running just ahead of the main component. $[\alpha]_D^{18°} + 2°$ (water, $c = 2{\cdot}1$). Found: C, 45·2; H, 8·3; N, 14·2; amino N (Van Slyke, 0·5 hr. reaction time), 9·5; Cl, 14·3. $C_{11}H_{23}O_3N_3.HCl.\frac{1}{2}H_2O$ requires C, 45·4; H, 8·6; N, 14·5; amino N, 9·7; Cl, 12·2%.

L-*Leucyl*-D-*phenylalanine*

From carbobenzoxy-L-leucine was prepared the acid chloride (Bergmann *et al.* 1936) by treating 0·25 g. with 0·25 g. PCl_5 in dry ether at 0°. When reaction was complete, the mixture was twice washed rapidly with equal volumes of cold water, and dried over Na_2SO_4. To this solution at 0° was added D-phenylalanine ethyl ester (prepared from 0·25 g. of its hydrochloride) in 4 ml. ethyl acetate. 3 ml. saturated aqueous $KHCO_3$ were added, and the mixture was kept at 0° with intermittent shaking for 1 hr. Water was added, and the organic solvent layer was separated and washed successively with N-HCl and 7% (w/v) $KHCO_3$ solution, dried, and concentrated *in vacuo*. The residue crystallized; recrystallization from ether-light petroleum yielded 0·22 g. *carbobenzoxy-L-leucyl-D-phenylalanine ethyl ester* suitable for further work. The product was recrystallized from chloroform-light petroleum, and had m.p. 103–105°, $[\alpha]_D^{17°} - 19°$ (ethanol, $c = 2{\cdot}7$). Found: C, 67·9; H, 7·1; N, 6·3. $C_{25}H_{32}O_5N_2$ requires C, 68·2; H, 7·3; N, 6·4%.

0·2 g. of this compound was dissolved in 1·5 ml. dioxan, and 1·5 ml. N-NaOH were added. The mixture, which became homogenous on gentle shaking, was kept for 45 min. at room temperature, and was then largely diluted with water and acidified. The oil which was precipitated was isolated by extraction with chloroform. The chloroform extract was evaporated to dryness *in vacuo*, yielding a syrupy residue of *carbobenzoxy-L-leucyl-D-phenylalanine*. This did not readily crystallize and was hydrogenated as described above. The gummy product was dissolved in 95% (v/v) aqueous ethanol; the solution was concentrated *in vacuo* until crystallization began, and ether was then added. In this way was obtained 0·12 g. of *L-leucyl-D-phenylalanine dihydrate*. A paper chromatogram with *n*-butanol showed only one band colouring with ninhydrin.

The product was recrystallized from warm 50% (v/v) aqueous ethanol. The water of crystallization was not readily lost in a vacuum desiccator at room temperature, but was removed on drying at 100° over P_2O_5 *in vacuo*. The hydrated material had $[\alpha]_D^{17°} + 25°$ (glacial acetic acid, $c = 2{\cdot}4$) and was employed for analysis. Found: C, 57·3; H, 7·9; N, 8·7; loss of weight on drying, 11·7. $C_{15}H_{22}O_3N_2.2H_2O$ requires: C, 57·3; H, 8·3; N, 8·9; loss of weight on drying, 11·5%. An amino-N determination (Van Slyke, 4 min. reaction time) on the material dried at 100° gave amino N, 5·0%; $C_{15}H_{22}O_3N_2$ requires 5·0%. Thus the water lost was solvent of crystallization, and cyclization to the diketopiperazine had not occurred.

D-*Phenylalanyl*-L-*proline*

0·4 g. of carbobenzoxy-D-phenylalanine was prepared from D-phenylalanine and converted to the acid chloride according to Smith & Brown (1941). The product was washed by decantation with dry ligroin and allowed to react with 0·3 g. L-proline dissolved in 4·4 ml. N-NaOH (cf. Bergmann *et al.* 1932). After 20 min. shaking at room temperature the still alkaline mixture was filtered, and the filtrate acidified (thymol blue) with 10N-HCl. An oil was precipitated, which hardened on keeping at 0° for a few hours. This material, presumed to be largely *carbobenzoxy*-D-*phenylalanyl*-L-*proline*, was filtered off and washed with water. Yield, 0·13 g. On attempting recrystallization, only rather greasy crystals were obtained. Accordingly, the whole was hydrogenated as described above. The reaction product was dissolved in ethanol. On addition of ether a flocculent precipitate resulted, which was filtered off, washed with ether, dried, and reprecipitated from the same solvents. The preparation had approximately the same optical rotation ($[\alpha]_D^{18°} - 93°$ and $-96°$, respectively, (acetic acid, $c = 2{\cdot}4$)) before and after reprecipitation. The final amorphous product (0·07 g.) proved, however, on paper chromatography with *n*-butanol, to be complex, giving a well marked faster moving band in addition to the stronger band corresponding to D-*phenylalanyl*-L-*proline*. The elementary analyses likewise depart considerably from the expected values; some of this deviation may, however, be due to retention of ethanol by the amorphous material dried at room temperature in a vacuum desiccator. Found: C, 62·4; H, 7·0; N, 8·3. $C_{14}H_{18}O_3N_2$ requires C, 64·1; H, 6·9; N, 10·7%. An attempt was made to prepare the acetyl derivative (cf. Behrens, Doherty & Bergmann, 1940) by acetylation with acetic anhydride and aqueous NaOH, in the usual way. Extraction of the acidified reaction mixture with *n*-butanol in chloroform (17% v/v), did not, however, yield satisfactory crystals.

L-*Prolyl*-L-*valine*

L-*Valine methyl ester hydrochloride* was prepared in nearly theoretical yield from L-valine by the methanolic HCl procedure used above. The reaction product crystallized on evaporation to dryness *in vacuo* and was recrystallized first from acetone-ether and subsequently from methanol-ether. The product, resulting in nearly theoretical yield, had m.p. 146–149°, $[\alpha]_D^{20°} + 14°$ (water, $c = 3{\cdot}0$) after drying in a vacuum desiccator at room temperature. Lower, and less sharp m.p.'s were observed with air-dry material, indicating the presence of solvent of crystallization. That some HCl, present as such, was incompletely removed even in the desiccator, is indicated by the analytical data for desiccator-dried material. Found: C, 41·8; H, 8·3; N, 8·2; Cl, 24·7. $C_6H_{13}O_2N.HCl$ requires C, 43·0; H, 8·4; N, 8·4; Cl, 21·2%.

Carbobenzoxy-L-proline was prepared according to Abderhalden & Nienburg (1933) from L-proline in 75% of the theoretical yield. 0·5 g. of the syrupy carbobenzoxy-L-proline was dissolved in 5 ml. dry ether and 0·5 g. PCl_5 was added to this solution at 0°. After shaking gently for 15 min. most of the PCl_5 had dissolved. The ethereal solution was decanted from undissolved PCl_5, and washed twice, quickly, with ice water and partially dried. To this solution was added an ethyl acetate solution of L-valine methyl ester, liberated from 0·4 g. of its hydrochloride by aqueous K_2CO_3. Saturated aqueous $KHCO_3$ was added to the mixture which was shaken intermittently during 20 min. After a further 40 min. water was added and the organic solvent layer was washed successively with N-HCl and 7% (w/v) aqueous $KHCO_3$, partially dried, and evaporated *in vacuo*, yielding 0·55 g. of colourless syrup. Attempts to bring this material, *carbobenzoxy*-L-*prolyl*-L-*valine methyl ester*, to crystallization were unsuccessful. 0·45 g. was saponified in 3 ml. dioxan with 3 ml. N-NaOH. The mixture quickly became homogeneous, and after 0·5 hr. at room temperature, was diluted with water. On acidifying (HCl), an oil was precipitated. This crystallized within a few hours. Yield, 0·33 g. of material suitable for subsequent use. *Carbobenzoxy*-L-*prolyl*-L-*valine* was recrystallized from methanol-water, and had m.p. 134–135°, $[\alpha]_D^{18°} - 58°$ (methanol, $c = 2{\cdot}8$). Found: C, 62·5; H, 6·9; N, 8·0; AEW, 350. $C_{18}H_{24}O_5N_2$ requires C, 62·1; H, 6·9; N, 8·0%; AEW, 348.

0·18 g. was hydrogenated as described above. 0·10 g. of crystalline material resulted. A paper-strip chromatogram developed with *n*-butanol revealed, on colouring with ninhydrin, a single yellow band. The material was recrystallized from water by addition of ethanol. Analysis showed the product to be L-*prolyl*-L-*valine hydrate*. The water of crystallization was lost very slowly in a vacuum desiccator at room temperature. $[\alpha]_D^{20°} - 61°$ (water, $c = 1{\cdot}7$) (calc. for the hydrate). The air-dry hydrate was used also for the analyses. Found: C, 52·1; H, 8·5; N, 12·6; amino N (Van Slyke, 4 min. reaction time), 0·0. $C_{10}H_{18}O_3N_2.H_2O$ requires C, 51·7; H, 8·6; N, 12·1%; amino N, nil.

SUMMARY

1. Syntheses are described of the dipeptides α-(L-valyl)-L-ornithine, L-ornithyl-L-leucine, L-leucyl-D-phenylalanine, D-phenylalanyl-L-proline and L-prolyl-L-valine. Carbobenzoxy derivatives were employed as intermediates, coupling being effected in each case through an acid chloride.

2. Some factors affecting the purity of the product in such syntheses are discussed.

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REFERENCES

Abderhalden, E. & Nienburg, H. (1933). *Fermentforschung*, **13**, 573.
Abderhalden, E. & Sickel, H. (1926). *Hoppe-Seyl. Z.* **152**, 95.
Abderhalden, E. & Spinner, H. (1919). *Hoppe-Seyl. Z.* **107**, 5.
Behrens, O. K., Doherty, D. G. & Bergmann, M. (1940). *J. biol. Chem.* **136**, 61.
Bergmann, M. (1935). *J. biol. Chem.* **110**, 471.
Bergmann, M., Zervas, L. & Fruton, J. S. (1936). *J. biol. Chem.* **115**, 593.
Bergmann, M., Zervas, L. & Ross, W. F. (1935). *J. biol. Chem.* **111**, 245.
Bergmann, M., Zervas, L., Schleich, H. & Leinert, F. (1932). *Hoppe-Seyl. Z.* **212**, 72.
Consden, R., Gordon, A. H. & Martin, A. J. P. (1944). *Biochem. J.* **38**, 224.
Consden, R., Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1947). *Biochem. J.* **41**, 596.
Cox, G. J. (1928). *J. biol. Chem.* **78**, 475.
Fischer, E. (1906). *Ber. dtsch. chem. Ges.* **39**, 2320.
Fischer, E. & Schoeller, W. (1907). *Liebigs Ann.* **357**, 1.
Fischer, E. & Warburg, O. (1905). *Ber. dtsch. chem. Ges.* **38**, 3997.
Fraenkel-Conrat, H. & Olcott, H. S. (1945). *J. biol. Chem.* **161**, 259.
Gillespie, H. B. & Snyder, H. R. (1943). *Organic Synth.* Coll. Vol. **2**, 489.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 79.
Hunter, A. (1939). *Biochem. J.* **33**, 27.
Kurtz, A. C. (1938). *J. biol. Chem.* **122**, 477.
Marvel, C. S. (1940). *Organic Synth.* **20**, 106.
Marvel, C. S. (1941). *Organic Synth.* **21**, 74.
Marvel, C. S. & du Vigneaud, V. (1943). *Organic Synth.* Coll. Vol. **2**, 93.
Neuberger, A. & Sanger, F. (1943). *Biochem. J.* **37**, 515.
Norris, J. F. (1907). *Amer. chem. J.* **38**, 627.
Sanger, F. (1946). *Biochem. J.* **40**, 261.
Smith, C. S. & Brown, A. E. (1941). *J. Amer. chem. Soc.* **63**, 2605.
Tristram, G. R. (1946). *Biochem. J.* **40**, 721.
Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627.
Willstätter, R. & Waldschmidt-Leitz, E. (1921). *Ber. dtsch. chem. Ges.* **54**, 113.

A Method for Estimating Peptic Activity in Gastric Contents

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An accurate simple method of estimating the activity of proteolytic enzymes is clearly desirable. To achieve this end, not only must a precise method of measuring proteolysis be devised, but a reproducible substrate must also be found. These two requirements were first met in the method of estimating pepsin described by Anson & Mirsky (1932) and Anson (1938). Their method, in which the substrate is carboxyhaemoglobin, has been widely used for estimating pure enzymes on account of its accuracy. In modified forms it has proved valuable in the determination of the activity of digestive juices (Beazell, Schmidt, Ivy & Monoghan, 1938; Bucher, Grossman & Ivy, 1945). However, without large scale apparatus, the preparation of sufficient quantities of the substrate of carboxyhaemoglobin is time-consuming and the method is correspondingly restricted in its applicability.

This paper describes the use of another reproducible but more readily available substrate. The modified reagents and procedure are given in full. A method of standardization is described and the adoption of a new pepsin unit is suggested.

METHOD

The substrate

General remarks. Dehydrated human plasma and serum, rejected as unfit for transfusion purposes, have been used exclusively for this work. The dried plasma or serum is of variable colour and is freely soluble in distilled water. Two samples have been encountered which immediately formed a gel on addition to water. These were discarded.

The substrate solution. Substrate solution is made up to contain 5·6 g. dried citrated plasma or dried serum in 100 ml. of distilled water with sufficient HCl to give an acidity of pH 2·1 as determined with a glass electrode at 18–22°. The substrate solution is stirred and then filtered through a Green's 309½ Agar filter paper or cotton wool. Considerable variations in the rate of filtration have been encountered but this had no apparent significance in the method.

Other reagents are: 0·350 N-trichloroacetic acid (approximately 6 g./100 ml.), 0·250 N-NaOH, HCl solution, pH 2·1, Folin & Ciocalteu's (1927) phenol reagent, phenol solution (5·0 mg./100 ml.).

Estimation of proteolytic activity

The sample of gastric juice under examination is mixed with an equal volume of dilute HCl of pH 2·1. A series of pairs of test tubes, one tube containing 5·0 ml. of substrate and the other 10·0 ml. trichloroacetic acid, are placed in a water bath at 35°. Stock tubes of gastric juice mixtures are also warmed in the water bath. After 20 min. 1·0 ml. of gastric juice mixture is added to the substrate tubes of pairs 1, 3, 5 *et seq.* and to the trichloroacetic acid tubes of pairs 2, 4, 6 *et seq.* Each addition is made at regular intervals of 30 sec. so that a series of up to 15 estimations can be handled in 14 min. Mixing is ensured by agitation of the tubes outside the water bath. Thus peptic digestion proceeds in the substrate tubes of the odd-numbered pairs whilst the even pairs provide controls.

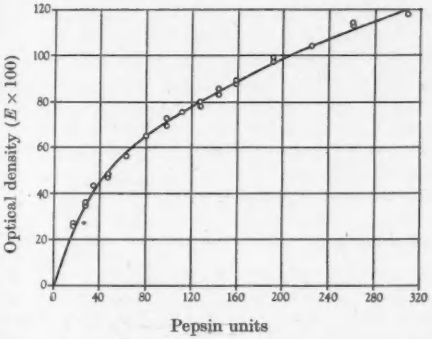

Fig. 1. The relationship between the difference in colour between control and test and the peptic activity of the gastric juice.

Exactly 15 min. after the addition of gastric juice to any pair of tubes, their contents are mixed by pouring back and forth. This stops any digestion in the odd-numbered pairs. After this mixing the tubes are allowed to stand in the water bath for approximately 4 min. The mixture is filtered through a 15 cm. paper (Whatman no. 1). 15–30 min. after the beginning of filtration 2·0 ml. of the filtrate are pipetted into a 50 ml. flask containing 20 ml. 0·25 N-NaOH and thorough mixing is ensured by swirling (vigorous shaking must be avoided as it tends to reduce the development of colour). Forthwith 1·0 ml. of Folin & Ciocalteu's phenol reagent is run down the side of the flask and mixed by immediate swirling.

A blue colour gradually develops and 15–40 min. later it is estimated in a photoelectric colorimeter with a suitable filter. A single cell colorimeter (King, 1946) with an Ilford 204 filter has been found satisfactory.

The difference in colour between the test and the control is used to evaluate the peptic activity from a curve which relates activity to colour difference (Fig. 1). If a more accurate evaluation is required

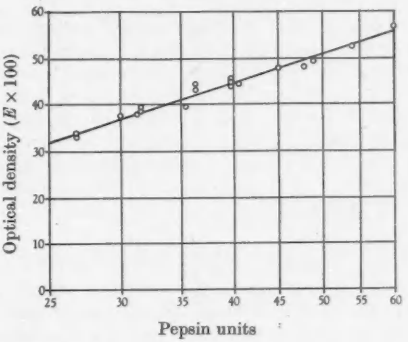

Fig. 2. The relationship between the difference in colour between control and test and the peptic activity of the gastric juice, plotted on a logarithmic scale.

Table 1. *Relation of peptic activity of gastric juice to difference in colour between control and test*

Pepsin units	Optical density ($E \times 100$) (Difference between control and test)
10	15·0
15	21·0
20	27·0
25	32·0
30	36·8
35	41·0
40	44·5
45	48·0
50	51·0
60	56·0
70	60·5
80	64·5
90	68·0
100	71·4
110	75·0
120	78·0
130	81·0
140	83·5
150	86·2
160	89·0
180	94·0
200	98·7
220	103·0
240	107·2
260	111·5
280	115·0
300	118·0

and the peptic activity falls between 25 and 60 pepsin units/ml. (see below), the straight-line relationship, in which the peptic activity is plotted on a logarithmic scale can be used (Fig. 2). These curves can be plotted from the values shown in Table 1.

A gastric juice is said to have 50 units of peptic activity/ml. when under the standard conditions of estimation (viz. after dilution with an equal part of HCl solution of pH 2·1) it gives a colour equivalent to that produced by 2·0 ml. of a standard phenol solution (5·0 mg./100 ml.).

The standard colour is developed by mixing 2·0 ml. of freshly prepared phenol solution (5·0 mg./100 ml.) with 20·0 ml. 0·25N-NaOH and promptly adding 1·0 ml. Folin & Ciocalteu's phenol reagent. Between 5–10 min. later the colour is read in the colorimeter. A blank estimation is made omitting the phenol; in this way ten consecutive estimations gave values, after subtracting the blanks, of 0·51 with no measurable deviations.

The relation of peptic activity to the colour produced

Since the method was devised to determine the proteolytic activity of gastric juice, this was used in preference to a solution of pure pepsin in establishing the relationship between proteolytic activity and colour differences between control and test. A series of known dilutions of a gastric juice were made. After 15 min. digestion, the value of the difference in colour between control and test for each of these dilutions was recorded. With these values as ordinates and percentage concentration of gastric juice as abscissae, a curve was plotted. In this way twelve curves relating peptic activity to the colour produced were made, two for juice obtained after the injection of histamine in known hypersecretory subjects and the remainder for fresh resting and post-prandial juice. The percentage concentration of gastric juice, corresponding to an ordinate value of 0·51 optical density, was then found from each curve and this percentage concentration was taken as equivalent to 50 pepsin units. The figures for percentage concentration of gastric juice were then converted into values in pepsin units/ml. for each curve. Thus if a concentration of 32% contained 50 pepsin units/ml. all the abscissal percentage concentrations for that particular curve were multiplied by $\frac{50}{32}$ to give values in pepsin units/ml. From the 12 recalculated curves a composite curve, based on 138 determinations, was plotted. The values of Table 1 are taken from the curve so produced.

The experimental points shown in Fig. 1 have been selected to include those furthest from the composite mean curve whilst all the determinations made over the range 25–60 pepsin units are shown in Fig. 2.

The method may be used in any laboratory by determining the colour given by the standard reagents with 2 ml. phenol solution (5·0 g./100 ml.). If this optical density be C, then before reading the peptic activity from the curve, all colorimeter readings must be corrected by a factor $C/0{\cdot}51$, since the graph is plotted for our equivalent of the standard, viz. 0·51 optical density.

One of the disadvantages of using gastric juice as a source of proteolytic enzyme, for establishing the relationship between colour produced and peptic activity, is that gastric juice of very high activity is difficult to obtain. With a solution of pepsin, concentrations greater than those found in gastric juice can be used to produce a curve whose range embraces all peptic activities likely to be encountered in gastric juices. In determining the relationship shown in Table 1, the experiments for values of above 150 pepsin units/ml. was made by increasing the digestion time. The validity of this step is supported by the fact that, provided the product of digestion time and dilution is constant, the colour produced is constant. No samples of gastric juice have been encountered with an activity falling outside the range of Table 1.

EXPERIMENTAL BASIS OF METHOD

The details of the preceding method are based upon a series of experiments and the following is a summary of their more important results. The gastric juice in the experiments described was obtained from pooled filtered fractional-test-meal samples adjusted to pH 2·1.

Concentration of substrate. Table 2 shows the effect of variation of concentration of the substrate on the values obtained for peptic activity for a single specimen of gastric juice. A concentration of 5·6 g./100 ml. has been chosen as being the highest concentration conveniently prepared from fresh citrated plasma which can be used in place of the dried product. If untreated plasma is used a different curve relating activity to colour is needed. A high concentration of plasma is desirable to ensure maximal buffering and to increase the range of peptic activity over which the method can be employed (see Beazell *et al.* 1938). If it is desired to husband the substrate, the method has been found to be quite satisfactory for juices of activity

Table 2. *Effect of variations of substrate concentration*

(Optical density ($E \times 100$) corresponding to peptic activity at different concentrations of substrate.)

		Conc. of substrate (g./100 ml.)				
		4	5	5·6	6	7
Activity of dilutions of a gastric juice in pepsin units	9·0	13·4	13·6	13·3	13·0	8·9
	31·5	33·0	35·5	37·8	35·3	35·5
	45·0	44·0	48·0	48·0	48·0	44·3
	63·0	52·0	59·5	57·5	59·5	55·9

Table 3. *Relation between the* pH *of substrate and enzyme activity*

pH	1·4	1·6	1·8	1·9	2·0	2·1	2·2	2·3	2·6	3·5
Optical density ($E \times 100$) corresponding to peptic activity with 3% substrate. Gastric juice 'P'	22·0	26·0	31·0	34·5	36·0	37·0	37·0	34·0	31·0	18·5
Optical density ($E \times 100$) corresponding to peptic activity with 5·6% substrate. Gastric juice 'Q'	—	—	—	48·5	51·0	51·2	51·0	48·8	—	—

up to 100 units/ml., with concentrations of 2·4 g. substrate/100 ml. and 4 g./100 ml. trichloroacetic acid. A new curve relating peptic activity to the colour produced is required for each such variation in substrate.

pH *of substrate.* Table 3 shows the relation between the pH of the substrate and rate of digestion for two specimens of gastric juice. The optimum pH lies between 2·0 and 2·2 and it can also be seen that this does not change with the concentration of the substrate.

Influence of variations of pH of gastric juice. Table 4 shows the effect of variations of the pH of a single sample of the gastric juice upon the results for the estimation of its peptic activity. From a 'pooled' gastric juice of pH 4·1 three dilutions were made to contain equal concentrations of the original juice at pH of 1·6, 2·1 and 4·1. These diluted

Table 4. *Effect of variations of* pH *of gastric juice on the estimation of its activity*

pH of gastric juice	1·6	2·1	4·1
Optical density ($E \times 100$) corresponding to peptic activity	8·7	8·6	8·8
pH of substrate (3 g./100 ml.) after adding diluted gastric juice	2·05	2·10	2·12

samples were then estimated by the routine method, with a substrate containing 3 g. plasma proteins/100 ml. A similar result was obtained with all these specimens. Further the pH of the substrate after the addition of the gastric juices was determined. It may be seen that this pH was not shifted outside the limits for the maximal rate of digestion. These experiments are of great practical importance because they show that it is not necessary to adjust the pH of the gastric juice to that of the substrate even when the latter is used in a dilute solution.

Physiological variables. It has been found that additions of chloride, bile or mucus do not influence the estimation of the activity of gastric juice.

Reproducibility of substrate. To test the reproducibility of the substrate, samples of protein were obtained from various sources (e.g. America and Britain) and used in the estimation of the activity of three samples of gastric juice. Table 5 shows that, despite the difference of origin of the materials employed, the results obtained were surprisingly consistent. Sample CP199/330, which gave a high result, had been shipped to Italy and back where conditions of storage may not have been favourable.

Stability of the substrate solution. The results of experiments made to test the stability of the substrate solution are shown in Table 6. Substrate solutions were made on the

Table 5. *The estimation of the activity of samples of gastric juice with proteins from various sources*

Protein substrate	Gastric juice	Optical density ($E \times 100$) corresponding to peptic activity: Individual values	Means
2 May			
Slough serum protein$_1$	A	31·5 31·0	31·3
Slough serum protein$_2$	A	28·4 29·4	28·9
British plasma protein	A	30·0 29·4	29·7
American plasma protein	A	30·2 29·4	29·8
11 July			
Plasma proteins LP119/6	B	39·2 39·2 39·8 39·8	39·5
Plasma proteins LP119/122	B	41·2 40·0 40·0 38·5	39·9
Plasma proteins LP119/4	B	39·0 40·0 39·0 40·0	39·5
Plasma proteins LP119/228	B	39·0 37·7 40·0 41·0	39·4
Plasma proteins CP199/330	B	41·8 41·0 42·0 41·5	41·6
Plasma proteins LP119/50	B	38·5 39·5 40·1 39·3	39·4
26 Sept.			
Plasma LP119/50	C	69·5 70·0 69·0 70·0	69·6
Serum 1050–46	C	69·0 69·0 70·0 69·0	69·3

dates shown and kept under the conditions shown. They were later concurrently used to estimate the peptic activity of a gastric juice. It is apparent that the substrate is stable for at least 24 hr. at 20° and for 48 hr. at 6°. The gastric juice has been found to be stable for approximately 6 hr. at 20° after dilution.

Table 6. *Tests of the stability of substrate*

Substrate solution	Date of test	Optical density ($E \times 100$) corresponding to peptic activity: Individual values	Mean
Made up immediately before test. 23 Mar.	23 Mar.	58·9 59·5	59·2
Made up 22 Mar. Kept at 20° for 24 hr.	23 Mar.	59·9 60·3	60·1
Made up 21 Mar. Kept 4 hr. at 20° followed by 48 hr. at 6° in refrigerator	23 Mar.	60·5 60·3	60·4

Trichloroacetic acid. The strength of the trichloroacetic acid has been chosen by trial and error to give a clear filtrate. It should be standardized by titration to 0·350 N.

The filtration. A very small amount of the substance producing blue colour is retained by the filter paper, but this is of little consequence as the error is minimized by the use of the curve relating activity to colour. The filtrate is not uniform, and therefore filtration should be allowed to proceed almost to completion as it does in the 15 min. allotted, and the filtrate must be mixed before the 2·0 ml. are pipetted out for analysis. The colour-producing property of the filtrate is only constant up to 30 min. after the beginning of filtration.

The colour-developing reaction. This is influenced by hydrogen ion concentration and by the amount of Folin & Ciocalteu's reagent used. If less alkali than the standard 20·0 ml. 0·25 N-NaOH is used the colour develops more slowly to a higher intensity, while with more than 20 ml. the colour develops more quickly but begins to fade sooner. Thus 20 ml. ensures a useful balance between the rate of development and the fading of the colour, which is maintained at a constant intensity for 25 min. under the standard conditions at pH 12·5. The rate of development and persistence of the colour is not influenced by the concentration of the filtrate.

If the colour produced is too dark to be read in the colorimeter, it can be diluted with a convenient volume of water before reading. The true colour can then be calculated.

1·0 ml. of undiluted Folin & Ciocalteu's reagent has been found to be convenient and, further, it appears that when this amount is used errors in measuring produce minimal changes in the final colour developed. The reagent should be admitted uniformly for all tests.

To test the reproducibility of the phenol reagent four samples were collected from various laboratories, where they were kept in refrigerators, and tested against the phenol standard and the Folin & Ciocalteu reagent in use for this work. In spite of the different degrees of yellowness of the samples they gave consistent results with the phenol standard (Table 7).

Table 7. *Test of the reproducibility of Folin & Ciocalteu's phenol reagent from different sources*

(2·0 ml. standard phenol (5·0 ml./100 ml.) were used in each test.)

Source of sample	Optical density ($E \times 100$): Phenol reagent and NaOH	Phenol reagent, NaOH and standard phenol	Difference corresponding to phenol standard
British Drug Houses Ltd. 646256/460909	2·0 2·0	52·5 52·5	50·5 50·5
Unknown	4·5 4·6	55·0 55·0	50·5 50·4
Hopkins and Williams Ltd. 1 Nov. 1946	2·0 2·0	53·0 53·0	51·0 51·0
British Drug Houses Ltd. 650319/470219	2·0 2·0	53·0 53·0	51·0 51·0
British Drug Houses Ltd. 643791/460424	2·0 2·0	53·0 53·0	51·0 51·0

Experiments have shown that it is essential to add the phenol reagent with the minimum of delay to obtain consistent results. The consistency obtainable by these procedures was tested by estimating ten samples from a pooled filtrate. The readings of these ten estimations fell between 22·8 and 23·0. It is particularly important that the 50 ml. flask used should be free from contamination with any colour-producing agent. As commercial ethanol contains such substances, it is advised that the final washing of these flasks should be with water.

Differences between duplicates may be due to such contamination, and this can be checked by analysis of another sample of the same filtrate. Substances which reduce the amount of colour produced have not been detected, therefore, if the duplicates differ, the lower reading is probably better.

In the original method of Folin & Ciocalteu (1927), Na_2CO_3 was used as the alkali in the final system. Anson & Mirsky (1932) used NaOH in place of Na_2CO_3 and this has been found the more reliable agent.

The accuracy of the method. Fourteen measurements of the activity of a single sample of gastric juice with one substrate gave a mean reading of 44·0 pepsin units, with a coefficient of variation for individual readings of $\pm 2{\cdot}64\%$. The coefficient of variation of an individual reading in pepsin units for twenty-eight determinations with seven samples of substrate shown in Table 5 for the experiments of 11 July and 26 September is $\pm 2{\cdot}5\%$. Results for sample CP 199/330 have been omitted (cf. p. 107).

DISCUSSION

The main principles of the method of Anson & Mirsky (1932) have been followed, but several significant details have been altered. They used tyrosine as a standard, but the appropriateness of this substance is perhaps doubtful since the digestive products giving a blue colour are probably a mixture of tyrosine and tryptophan. What is needed is a stable substance which can be readily obtained in

a pure state, and which will produce a consistent colour with the Folin & Ciocalteu reagent. Phenol is such a substance and it has been used to provide the colour standard in this work. Thus the preparation of a pure sample of tyrosine is avoided.

Since the relationship between concentration of phenol and the colour produced in the reaction with the phenol reagent is not linear over a wide range, peptic activity has been related to a standard phenol at one point only. A small divergence from true proportionality is also found for the mixture of tyrosine and tryptophan estimated in the digestion products. Herbert (1946) has confirmed this point for the estimation of phenol with Folin & Ciocalteu's phenol reagent when $NaCO_3$ is used as the alkaline medium for colour development.

The method has proved quite practicable with ordinary citrated plasma so that, in the absence of a supply of dried plasma or serum, it could be used with citrated or whipped ox blood. However, it is unlikely that the supply of plasma or serum will fail, since occasional batches will always be rejected as unfit for transfusion because they are icterogenic. A new relationship between peptic activity and colour would, however, have to be established for fresh plasma. Any alteration of the concentration of the substrate beyond the range 5–6 g./100 ml. would also require a simultaneous modification of the strength of the trichloroacetic acid to ensure that the optimal pH was reached in the colour development reaction.

The method described estimates the total proteolytic activity of gastric juice at pH 2·1 with one special substrate. The practice is to attribute all this activity to pepsin, and the method has therefore been described as a method for measuring pepsin. However, the work of Northrop (1939) suggests that it may later prove necessary to apportion the activity of gastric juice to pepsin, gelatinase and possibly other enzymes. The uncertainty surrounding the source of the activity of the gastric juice seemed in part to justify the completely arbitrary measure of proteolytic activity used in this work.

SUMMARY

1. A method is described for measuring the proteolytic activity of gastric juice with a substrate of dried plasma or serum protein which has been found to be reproducible and convenient.

2. The method allows of the comparison of peptic activity in different laboratories without reference to a standard pepsin.

REFERENCES

Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Anson, M. L. & Mirsky, A. E. (1932). *J. gen. Physiol.* **16**, 59.
Beazell, J. M., Schmidt, C. R., Ivy, A. C. & Monoghan, J. F. (1938). *Amer. J. digest. Dis.* **5**, 661.
Bucher, G. L., Grossman, M. I. & Ivy, A. C. (1945). *Gastroenterol.* **5**, 501.
Folin, D. & Ciocalteu, V. (1927). *J. biol. Chem.* **73**, 627.
Herbert, F. K. (1946). *Quart. J. Med.* **15**, 221.
King, E. J. (1946). *Microanalysis in Medical Biochemistry.* London. Churchill.
Northrop, J. H. (1939). *Crystalline Enzymes.* New York: Columbia University Press.

The Persistence in the Blood Stream of some Compounds Related to Suramin

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Suramin (Antrypol, Germanin, Bayer 205, Moranyl, F. 309, Belganyl, Naganol, see formula I) displays an extraordinarily marked persistence in the blood stream of man and experimental animals. This property was recognized soon after suramin was first discovered, first, from the long duration of its prophylactic action (Mayer & Zeiss, 1920; Levaditi & Klarenbeek, 1927); secondly, from measurements of the trypanocidal activity of serum and urine of treated animals (Mayer, 1922; Mayer & Menk, 1922). The development of adequate chemical methods of determining it (Steppuhn & Utkin-Ljubovzov, 1924; Lang, 1931; Dangerfield, Gaunt & Wormall, 1938*a*; Boursnell, Dangerfield & Wormall, 1939; Vierthaler & Boselli, 1939), and their use in studying the persistence quantitatively (Lang, 1931; Dangerfield, Gaunt & Wormall, 1938*b*; Boursnell, *et al.* 1939; Vierthaler & Boselli, 1939; Hawking, 1940*a*, *b*) showed that in man, dogs and rabbits, it may persist in the blood stream for as long as 3 months. The

form of the blood concentration-time curve is asymptotic (Lang, 1931); during the first few hours after intravenous administration the concentration falls rapidly, but subsequently more and more slowly, until it may be regarded as almost constant over periods of a few days.

Some work has been done on the biochemical and physicochemical characteristics of suramin responsible for this remarkable persistence. No depot of suramin appears to be formed in any tissue. In general, the tissue concentrations are of the same order as, or lower than, the plasma concentration (Zeiss & Utkina-Ljubovtzova, 1930; Boursnell & Wormall, 1939). It must therefore be assumed that the kidney is able to excrete suramin only very slowly, although it can be detected in the urine during the first few days after dosing (Boursnell *et al.* 1939). The most widely held theory advanced to account for this slow excretion is that suramin is bound to protein, either in plasma or cells. Mandel & Steudel (1926) suggested that it was combined in the body with basic proteins of the histone type. They demonstrated that such combination could occur *in vitro*. Collier (1926) showed that suramin in 1% (w/v) concentration could protect horse serum from heat coagulation, and that it combined with globulins (Collier, 1926, 1927). Brunelli (1934) showed that it combined with the euglobulin fraction of horse serum and, to a less extent, with the pseudoglobulin fraction. Boursnell & Wormall (1939) considered that proteins other than the globulins might also be involved; and Dewey & Wormall (1946) demonstrated the combination *in vitro* of suramin with proteins of various types. They precipitated the protein with alcohol in the presence of suramin, and then extracted the protein-suramin complex with ethanol in a Soxhlet apparatus. Large amounts of suramin remained bound to the protein, even after this treatment. It seems likely that the persistence is largely due to this firm combination with the body proteins; however, other factors may be involved. Many drugs that are largely bound to protein are rapidly eliminated, their slow filtration through the glomeruli being augmented by participation of the tubular epithelium in the renal excretory processes. Examples are sulphathiazole, *p*-aminobenzoic acid (Lundquist, 1945) and penicillin (Beyer, Peters, Woodward & Verwey, 1944; Rantz, Kirby & Randall, 1944; Beyer, 1947). Conversely, the most persistent sulphonamides are those that are extensively reabsorbed from the glomerular filtrate by the tubular epithelium (Fisher, Troast, Waterhouse & Shannon, 1943; Earle, 1944). Usually they are also bound to plasma protein. Tubular processes may possibly play a similar part in the persistence of suramin. The strength of the bond uniting it to protein may also be of importance (Gregerson & Rawson, 1943; Rawson, 1943). A further possibility, which does not seem to have received attention, is that suramin exists in solution as colloidal aggregates of sufficiently high nominal molecular weight to hinder passage through the glomerulus. Some support is provided by the known inability of suramin to pass through a collodion membrane (Boursnell *et al.* 1939).

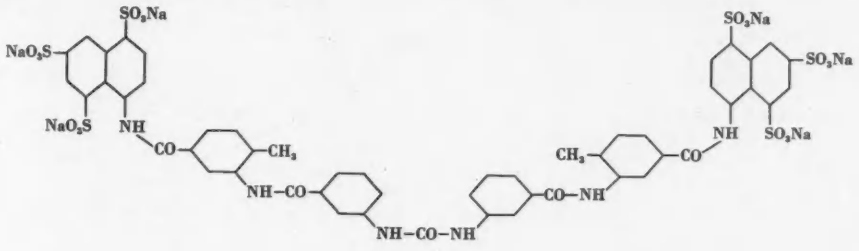
The high persistence of suramin raises interesting questions regarding its degradation by the body. No drug so far adequately examined has proved to be completely unaffected by metabolic processes. If suramin is degraded it must be assumed, either that the process involved is extremely slow, or that the products are themselves highly persistent. The prophylactic action of suramin is associated with the maintenance of a definite blood concentration of about 1·3 mg./100 ml., as determined by the Wormall method (Vierthaler & Boselli, 1939). It is possible that part of this concentration may be contributed by degradation products. The most obvious method of degradation of suramin, a polyamide related in structure to the natural polypeptides, is by hydrolysis to the three constituent aromatic amino-acid residues. The possibility that these are formed *in vivo* was considered by Dewey & Wormall (1946) who showed that a mixture of them, prepared by acid hydrolysis of suramin, was rapidly eliminated. If the postulated degradation gives the constituent amino-acids, it must proceed very slowly. The further possibility that degradation might stop at an intervening stage, yielding a persistent urea or amide, was not examined.

It seemed to the author that an examination of the persistence of compounds structurally related to suramin might provide information on three main problems of interest. First, it might indicate the structural features of the suramin molecule that confer high persistence. Such information would be of general value in the synthesis of prophylactic drugs. Secondly, it might show whether the structural features conferring high persistence are also those conferring high activity. If this were so it could be tentatively assumed that the activity of suramin involves union with an enzyme of the parasite, qualitatively similar to its union with the plasma proteins. Thirdly, if potential hydrolysis products of suramin were examined, their persistence would indicate whether they were likely to be formed *in vivo*.

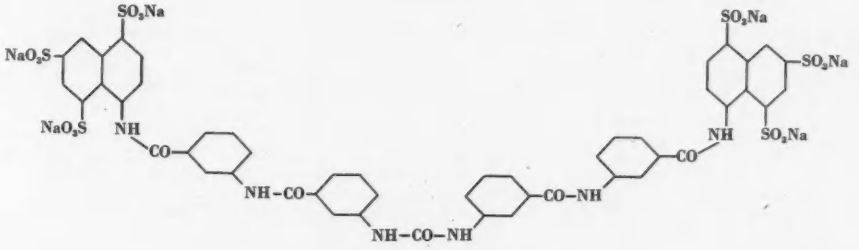
EXPERIMENTAL

Twelve compounds in all were examined. Their structural formulae are shown below. Since they are highly complex, they are referred to throughout by code numbers. Most of them were prepared in these laboratories by Dr R. A. Wilkinson, to whom the author is very grateful.

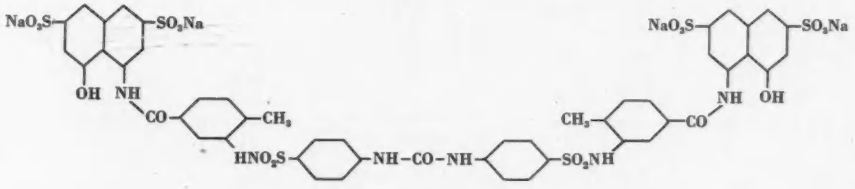
Three methods were used for determining the compounds, based on the standard techniques of diazotization and coupling used in these laboratories



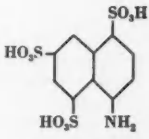
I (suramin)



II (demethylated suramin)

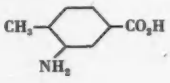


III

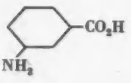


(as a mixed disodium salt)

IV



V



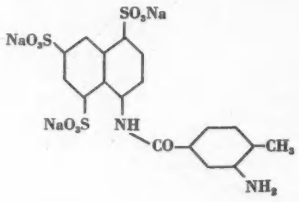
VI

be devised. When hydrolysis was necessary, alkaline conditions were used, because the final colour given by normal plasma was thereby reduced. Since some

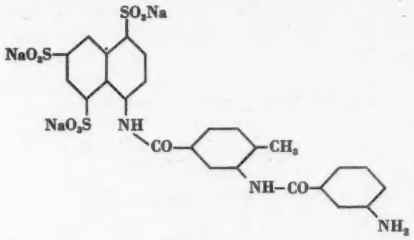
(Rose & Bevan, 1944; Rose & Spinks, 1946; Spinks & Tottey, 1946). They represent the first sets of conditions tried that gave adequate (±10%) recoveries, and are probably not the best that could

of the compounds were rapidly eliminated, this advantage outweighed those of acid hydrolysis described by Dangerfield *et al.* (1938*a*). It should be particularly noted that no attempt was made to

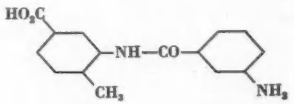
discover the shortest possible time of hydrolysis; 6 hr. was regarded as ample time and used in all experiments.



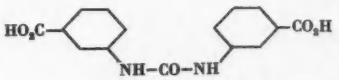
VII



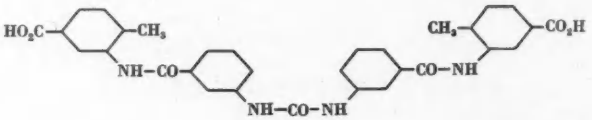
VIII



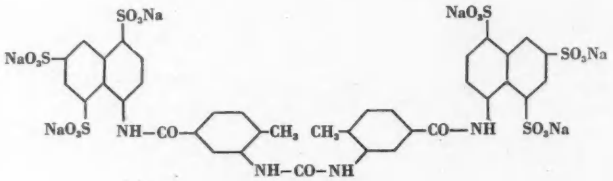
IX



X



XI



XII

Method A, used for compounds V, VI, VII, VIII and IX

Plasma (0·2 ml.) was pipetted into 2 ml. of water in a tube graduated at 10 ml., and treated with 2 ml. of 15% (w/v) trichloroacetic acid. The tube was shaken, allowed to stand for 5 min. and centrifuged at 2000 r.p.m. for 10 min., 0·5 ml. of 0·1% (w/v) $NaNO_2$ was then added; the solutions were well mixed, and adhering protein was removed from the walls of the tube with a thin rod. The tube was then recentrifuged for 5 min. Exactly 20 min. after adding nitrite, 0·5 ml. of 1% (w/v) *N*-β-sulphatoethyl-*m*-toluidine and 2 ml. of 30% (w/v) sodium acetate.$3H_2O$ were added, and the solution was well mixed without disturbing the protein clot. After 20 min. 0·5 ml. of conc. HCl was added, and the volume was made up to the mark with distilled water. The solution was again stirred and the tube was recentrifuged for 5 min. The colour was then read in a photoelectric colorimeter against a series of standards similarly prepared from 0·2 ml. volumes of aqueous solutions containing 0–50 mg. of the appropriate compound/100 ml.

Notes. Coupling of each diazonium compound was approximately complete after 20 min. under the conditions described. A 1 cm. cell and a blue-green filter were used in the colorimeter.

Method B, used for compound IV

Method B was identical with method A except that coupling was allowed to proceed for 40 min. after adding acetate. Even then it was less than 50% complete. The

slow coupling of diazotized 1-naphthylamine-4:6:8-trisulphonic acid (IV) is probably due to steric hindrance by the bulky group in the 8-position. The colours produced were very weak. A 2 cm. cell and a green filter were used in the colorimeter.

Method C, used for compounds I, II, III, X, XI and XII

Plasma (1 ml.) was treated with 2 ml. of 2N-NaOH in a tube graduated at 10 ml.; the tube was stoppered with a vaselined glass stopper, and heated at 100° for 6 hr. After cooling, 1 ml. of conc. HCl was added, and the volume was made up to 10 ml. with distilled water; 2 g. of kaolin (B.P.) were then added, and the tube was well shaken for 1 min. The contents were filtered, and 2 ml. of the almost colourless filtrate were treated with 0·5 ml. of 0·1% (w/v) $NaNO_2$ in a tube graduated at 10 ml. After 20 min. 0·5 ml. of 1% (w/v) *N*-*β*-sulphatoethyl-*m*-toluidine and 1 ml. of 30% (w/v) sodium acetate ($3H_2O$) were added. Coupling was allowed to proceed for 20 min., and 0·5 ml. of conc. HCl was then added. The volume was made up to 10 ml. with distilled water, and the sample was read in the colorimeter against standards similarly prepared from 1 ml. volumes of aqueous solutions of the appropriate compound, ranging in strength from 0 to 50 mg./100 ml.

Notes. Alkaline hydrolysis gave lower blanks from normal plasma than acid hydrolysis. No kaolin was added to the standards. A 1 cm. cell and a blue-green filter were used in the colorimeter.

Recoveries of compounds from plasma. Recoveries of known amounts of the compounds from plasma are shown in Table 1.

Table 1. *Recoveries of known amounts of compounds from plasma*

Compound*	Added (mg./100 ml.)	Found (mg./100 ml.)	Recovery (%)
I	5·0	5·5	110
	10·0	8·5	85
	20·0	22·0	110
	30·0	32·5	108
	50·0	46·5	93
II	10·0	10·4	104
	15·0	14·0	93
	20·0	21·7	108
	30·0	26·9	90
	40·0	38·5	96
	50·0	51·4	103
III	5·0	4·7	94
	10·0	9·1	91
	15·0	13·3	89
	20·0	18·3	91
	25·0	22·7	91
	30·0	23·9	80
	50·0	49·7	99
IV	5·0	5·5	110
	5·0	5·0	100
	15·0	13·5	90
	15·0	16·0	107
	25·0	22·5	90
	50·0	47·7	95
V	2·5	2·5	100
	5·0	5·5	110
	7·5	7·7	103
	12·5	12·5	100
	17·5	17·7	101
	25·0	23·8	95
VI	2·5	2·25	90
	2·5	2·5	100
	5·0	5·5	110
	7·5	8·2	109
	12·5	12·0	96
	15·0	15·7	105
	17·5	18·5	106
	17·5	18·5	106
	25·0	28·0	112
	25·0	23·0	92
VII	5·0	5·0	100
	15·0	17·0	113
	25·0	25·0	100
	25·0	26·1	104
	35·0	36·5	104
	50·0	53·5	107
VIII	10·0	15·0	150
	20·0	21·0	105
	30·0	29·5	98
	50·0	49·0	98
	70·0	66·0	94
	100·0	94·0	94
IX	5·0	5·0	100
	5·0	5·5	110
	10·0	10·5	105
	10·0	10·3	103
	15·0	15·0	100
	25·0	25·0	100
	25·0	27·0	108
	35·0	38·7	111
X	5·0	4·9	98
	10·0	9·7	97
	15·0	14·5	97
	20·0	18·4	92
	30·0	27·9	93
	50·0	49·5	99
XI	5·0	4·85	97
	10·0	12·1	121
	15·0	16·1	107
	20·0	20·8	104
	30·0	35·0	117
	40·0	45·2	113
XII	5·0	5·0	100
	10·0	8·6	86
	15·0	12·9	86
	20·0	15·2	76
	30·0	24·1	80
	50·0	43·5	87

* See p. 112.

Biological tests

Each compound was injected into a marginal ear vein of an adult rabbit in a dose of 100 mg./kg., as a 2·5, 5 or 10% solution of the sodium salt. Oxalated blood samples were taken at intervals from the marginal vein of the other ear, or by cardiac

puncture, and immediately centrifuged. The compound was then determined in the plasma by the appropriate method. From this preliminary experiment the approximate degree of persistence of the compound was calculated. It was then administered under the same conditions to a group of three rabbits, blood samples being withdrawn at standard intervals indicated by the preliminary experiment. From the plasma concentrations in the three rabbits a mean plasma concentration-time curve was constructed.

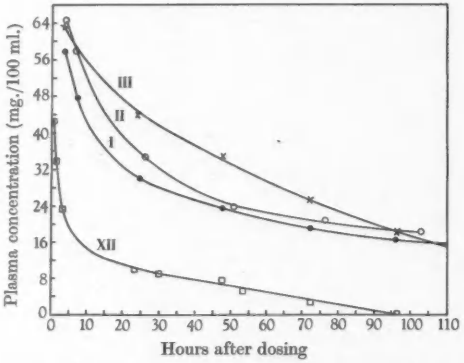


Fig. 1. Mean plasma concentration-time curves of compounds I, II, III and XII following the intravenous administration of 100 mg./kg. to groups of three rabbits.

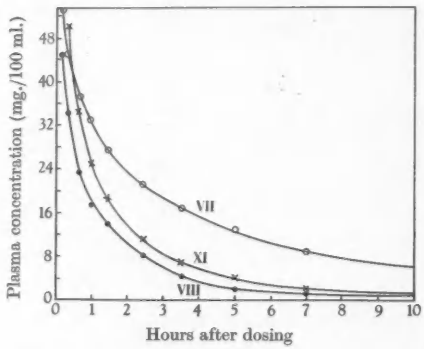


Fig. 2. Mean plasma concentration-time curves of compounds VII, VIII and XI following the intravenous administration of 100 mg./kg. to groups of three rabbits.

RESULTS

The mean plasma concentration-time curves of the twelve compounds are shown in Figs. 1–4. Since the compounds differed widely in persistence the scales of the four curves also differ widely, and should be carefully noted. The compounds are arranged in approximate order of decreasing persistence; this order is: I, II, III; XII; VII, XI, VIII; IV, IX, X, VI, V. The first group, including suramin (I), 'demethylated' suramin (II), and a closely related

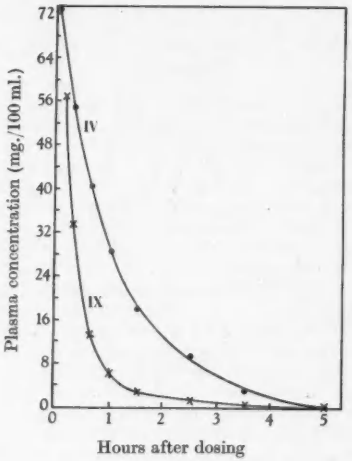


Fig. 3. Mean plasma concentration-time curves of compounds IV and IX following the intravenous administration of 100 mg./kg. to groups of three rabbits.

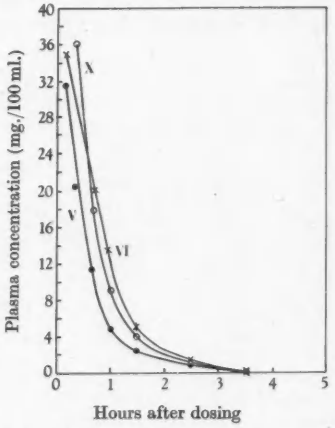


Fig. 4. Mean plasma concentration-time curves of compounds V, VI and X following the intravenous administration of 100 mg./kg. to groups of three rabbits.

compound (III), having H acid as an end group instead of naphthylaminetrisulphonic acid, and sulphanilamide as a linking group instead of *m*-aminobenzamide, persists in the blood stream for extended periods. After 5 days all three compounds are still present in the plasma at concentrations of about

16 mg./100 ml. The suramin curve is almost identical with that obtained by Dangerfield *et al.* (1938*b*) under similar conditions. The forms of the other two curves suggest that 'demethylated' suramin is very similar to the parent compound, and that the H acid derivative may be slightly less persistent. Compound XII, an analogue of suramin lacking two aminobenzoic acid links, is intermediate in persistence between the first group and the other, more rapidly eliminated, compounds. It is strongly persistent by ordinary standards, being detectable in the blood stream for 3–4 days. The third group of compounds (VII, VIII and XI) is rather weakly persistent, of about the same order as sulphadiazine (cf. Rose & Spinks, 1946). Each is detectable in the blood stream for 10–24 hr. The remaining five compounds (IV, V, VI, IX and X) are very rapidly eliminated, 1-naphthylamine-4:6:8-trisulphonic acid (IV) somewhat less so than the others. The results on IV, V and VI agree with those of Dewey & Wormall (1946) who examined a mixture of the three obtained by hydrolysis of suramin.

DISCUSSION

The results will be discussed in relation to the three problems outlined in the introduction.

1. *Persistence of suramin in relation to molecular structure*

The results show two main trends. First, compounds containing naphthalenepolysulphonic acids as end groups are more persistent than those containing carboxylic acids as end groups (cf. XI and XII; VII and IX; IV and V). Secondly, persistence tends to increase with increase in molecular weight (cf. XII and I, X and XI). From these two trends, and from individual characteristics of the twelve compounds, the following tentative deductions can be made. First, persistence of the suramin order is displayed only by polyamides containing 6 amino-acid groups (I, II, III as compared with XII). Secondly, high persistence is displayed only by polyamides containing naphthalenepolysulphonic acid groups (I, II, III, XII, VII, as compared with IX, X, XI). Thirdly, the presence of two naphthalenepolysulphonic acid groups appears essential for high persistence (XII as compared with VIII). Fourthly, *m*-amino-*p*-toluic, *m*-aminobenzoic and sulphanilic acid groups may be interchanged in the molecule without marked effect on the persistence (I, II, III). Fifthly, a phenolic group may be introduced without marked effect on the persistence (III). Sixthly, the shape and spacing of the molecule may be much modified without marked effect on the persistence (III).

All these deductions taken together strongly suggest that any symmetrical compound of high molecular weight containing a polysulphonic acid as an end group would show strong persistence. This view is supported by the rather strong persistence of some azo dyes of high molecular weight derived from naphthalenesulphonic acids (cf. Evans Blue, Trypan Blue; Dawson, Evans & Whipple, 1920). The susceptibility of the azo linkage to enzymic reduction is possibly what prevents their being retained in the body as long as suramin.

The fact that the spacing of the molecule does not appear to be of prime importance was not foreseen. It had been tentatively assumed that the persistence was due to the association of both polysulphonic acid groups with basic groups of a protein molecule. The difference in shape between I and III was therefore expected to result in reduced persistence of III. However, it may be that the two compounds combine with different proteins, or at different points of the same protein; and combination of compound with protein through the amide groups might also be possible. In that case, however, the dicarboxylic acid XI would be expected to show higher persistence.

2. *Persistence of suramin in relation to activity*

The low activity of II (Davey, 1943) and the trace activity of III (Browning, 1939), which are both of similar persistence to suramin, show that persistence and activity are due to different structural features of the molecule. The inactivity of many compounds prepared by Fourneau, Trèfouel, Trèfouel & Vallée (1924) which, from these results, would almost certainly be highly persistent, supports this view.

3. *Persistence of suramin in relation to its metabolism*

Compounds IV, V, VI, VII, VIII, IX, X, and XI are the chief potential hydrolysis products of suramin. Each is either very rapidly, or rather rapidly, eliminated. Other possible hydrolysis products, including asymmetrical ureas, would probably behave very similarly (cf. VIII, XI, XII). It can therefore be assumed that suramin either is not hydrolyzed *in vivo* or is hydrolyzed extremely slowly. The latter possibility appears rather unlikely, and the former may therefore be tentatively accepted. Other possible routes of metabolism could, however, be suggested which would probably give compounds of similar persistence to suramin, e.g. the oxidation of the latter to phenols, or phenolic sulphates or glucuronides. The long duration of the prophylactic action of suramin, and the known delicacy of the balance between structure and activity (Fourneau *et al.* 1924) strongly suggest that suramin is relatively resistant to metabolic processes.

SUMMARY

1. The persistence of suramin and eleven related compounds in the blood of rabbits has been investigated following their intravenous administration under standard conditions.

2. Marked persistence is a property of polyamides of high molecular weight that contain naphthylaminepolysulphonic acids as end groups.

3. Carboxylic acids of similar molecular complexity are more rapidly eliminated.

4. There is no relation between persistence and therapeutic activity.

5. Suramin is probably not hydrolyzed *in vivo*.

6. Methods of determining each of the twelve compounds in plasma are described.

The author wishes to thank Miss E. France and Miss R. B. Horrocks for valuable technical assistance.

REFERENCES

Beyer, K. H. (1947). *Science*, **105**, 94.
Beyer, K. H., Peters, L., Woodward, R. & Verwey, W. F. (1944). *J. Pharmacol.* **82**, 310.
Boursnell, J. C., Dangerfield, W. G. & Wormall, A. (1939). *Biochem. J.* **33**, 81.
Boursnell, J. C. & Wormall, A. (1939). *Biochem. J.* **33**, 1191.
Browning, C. H. (1939). Private communication.
Brunelli, B. (1934). *Arch. farmacol. sper.* **57**, 186.
Collier, W. A. (1926). *Rev. sud-amer. Endocrin.* **9**, 179.
Collier, W. A. (1927). *Z. ges. exp. Med.* **54**, 606.
Dangerfield, W. G., Gaunt, W. E. & Wormall, A. (1938*a*). *Biochem. J.* **32**, 59.
Dangerfield, W. G., Gaunt, W. E. & Wormall, A. (1938*b*). *Biochem. J.* **32**, 65.
Davey, D. G. (1943). Private communication.
Dawson, A. B., Evans, H. M. & Whipple, G. H. (1920). *Amer. J. Physiol.* **51**, 232.
Dewey, H. M. & Wormall, A. (1946). *Biochem. J.* **40**, 119.
Earle, D. P. (1944). *J. clin. Invest.* **23**, 914.
Fisher, S. H., Troast, L., Waterhouse, A. & Shannon, J. A. (1943). *J. Pharmacol.* **79**, 373.
Fourneau, E., Trèfouel, J., Trèfouel, J. & Vallée, J. (1924). *Ann. Inst. Pasteur*, **38**, 81.
Gregerson, M. I. & Rawson, R. A. (1943). *Amer. J. Physiol.* **138**, 698.
Hawking, F. (1940*a*). *Trans. Roy. Soc. Trop. Med. Hyg.* **34**, 37.
Hawking, F. (1940*b*). *Trans. Roy. Soc. Trop. Med. Hyg.* **34**, 217.
Lang, K. (1931). *Arch. exp. Path. Pharmak.* **160**, 560.
Levaditi, C. & Klarenbeek, A. (1927). *C.R. Acad. Sci., Paris*, **184**, 1213.
Lundquist, F. (1945). *Acta Pharmacol. Toxicol.* **1**, 307.
Mandel, J. A. & Steudel, H. (1926). *Hoppe-Seyl. Z.* **160**, 91.
Mayer, M. (1922). *Arch. Schiffs- u. Tropenhyg.* **26**, 237.
Mayer, M. & Menk, W. (1922). *Arch. Schiffs- u. Tropenhyg.* **26**, 208.
Mayer, M. & Zeiss, H. (1920). *Arch. Schiffs- u. Tropenhyg.* **24**, 257.
Rantz, L. A., Kirby, W. M. M. & Randall, E. (1944). *J. clin. Invest.* **23**, 789.
Rawson, R. A. (1943). *Amer. J. Physiol.* **138**, 708.
Rose, F. L. & Bevan, H. G. L. (1944). *Biochem. J.* **38**, 116.
Rose, F. L. & Spinks, A. (1946). *J. Pharmacol.* **86**, 264.
Spinks, A. & Tottey, M. M. (1946). *Ann. trop. Med. Parasit.* **40**, 101.
Steppuhn, O. & Utkin-Ljubovzov, X. (1924). *Klin. Wschr.* **3**, 154.
Vierthaler, R. W. & Boselli, A. (1939). *Arch. Schiffs- u. Tropenhyg.* **43**, 149.
Zeiss, H. & Utkina-Ljubovtzova, K. (1930). *Z. Immun-Forsch.* **68**, 170.

The Action of Chymotrypsin and Trypsin on Insulin

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Of the various chemical methods used in attempts to elucidate the structure of proteins, the breakdown by mild hydrolysis seems most promising. The constitution of the resulting fragments which can be isolated may then be ascertained separately. This method has been used to some extent by Gordon, Martin & Synge (1941, 1943) who identified a considerable number of small peptide fragments after partial hydrolysis with hydrochloric acid. Whilst the method undoubtedly throws some light on the order of amino-acids in the protein, a complete structural analysis would be a formidable, if not impossible, task, although it is capable of elucidating the structure of the pentapeptide gramicidin S (Synge, 1944, 1945; Consden, Gordon, Martin & Synge, 1946) and is no doubt capable of considerable extension. A more promising method in the case of a protein is hydrolysis by proteolytic enzymes into larger peptides which can then be further examined.

The essential conditions to be satisfied are: (1) The amount of enzyme added shall not be sufficient to contaminate the digest appreciably. This implies that both enzyme and its substrate must not be contaminated with foreign proteins. (2) No rearrangement of the amino-acids must be brought about by the action of the enzyme. Although synthetic actions of papain have been reported by Behrens & Bergmann (1939), there is at present no evidence that proteolysis brought about by animal proteinases such as pepsin and trypsin is a reversible process. We believe that the isolation of a peptide would provide powerful evidence of its existence as a structural component of the protein.

The work reported here is a preliminary examination of the action of the two trypsins on insulin. Numerous studies of the action of preparations of these enzymes on insulin have been made (for bibliographies see Jensen, 1938; Hill & Howitt, 1936), but much of this work is difficult to interpret, owing to the lack of adequate characterization of the enzyme preparations employed. Moreover, when considerable quantities of impure enzyme have been added, as has been done in some of these experiments, it is impossible to tell whether the split-products are derived from insulin or from foreign proteins accompanying the enzyme.

EXPERIMENTAL

Materials. Trypsin and chymotrypsin were prepared from frozen beef pancreas by the methods of Northrop & Kunitz (1932) and Kunitz & Northrop (1935, 1936), the chief modification being that trypsinogen was converted to trypsin and crystallized by the more recent method of McDonald & Kunitz (1946). The trypsin was only once crystallized, the quantity available being rather small. The chymotrypsin was stored in the form of chymotrypsinogen (eight times recrystallized). When required it was activated by the addition of a very small quantity of trypsin (<0·5%) and adjusting the pH to 7·6.

Insulin was a crystalline product prepared by Boots Pure Drug Co. Ltd. A portion was recrystallized three times by dissolving 1 g. in water (170 ml.) containing glacial acetic acid (3 ml.). A solution containing 1 mg. of Zn^{++} in the form of its acetate was added, and the pH was adjusted to 6·6 with ammonia. After dilution to 340 ml., the pH was adjusted to 5·9 with glacial acetic acid, and crystallization took place in less than 1 hr. No significant differences were found between the original and the recrystallized insulin, except that the latter contained a rather higher percentage of non-protein N; the material was not dried by washing with ethanol and ether and therefore contained some mother-liquor.

METHODS

Non-protein N (N.P.N.). This was determined by adding 1 ml. of digest to 0·3 N-trichloroacetic acid (5 ml.) at room temperature, allowing to stand for 0·5 hr. and filtering. A micro-Kjeldahl N determination was performed on the filtrate.

Amino N. The sample (1 ml.) was added to 5 ml. of a buffer solution having a pH suitable for arresting enzyme action, i.e. to 0·1 N-acetic acid in the trypsin and chymotrypsin experiments and 0·1 N-phosphate buffer of pH 7·5 in the case of pepsin. The amino N in a portion of the solution was determined by the Van Slyke method in the usual manner. Sanger (1945) has shown that terminal glycyl residues give an abnormally high value for amino N under the conditions used in the Van Slyke method. Since the terminal residues of the peptides are unknown, no correction has been made, but their characterization largely as glycine residues would introduce a significant error in our estimates of the number of peptide bonds broken.

Total N (T.N.). Three hours' digestion with 1 ml. conc. H_2SO_4 containing about 50 mg. of $CuSO_4$, 50 mg. of K_2SO_4 and a few mg. of Na_2SeO_4 was used. With insulin very little change in the T.N. was observed when the period of digestion varied between 2 and 17 hr.

Free amino-acids. These were determined by the ninhydrin method of Van Slyke, Dillon, MacFadyen & Hamilton (1941). Some difficulty was experienced in getting concordant values, the amount of free amino-acid being so small as to give readings of the same order of magnitude as the blanks.

Diffusion coefficient. A cell of the Anson-Northrop type with a sintered-glass membrane was employed. The cell constant, as determined with 0·1-N KCl, was 1·79 cm.^{2} (mean of three results, two before and one after the actual diffusion experiments). The technique used was similar to that described by Mehl & Schmidt (1937). D_{25} (0·1 N-KCl) was taken as 1·46 cm.^{2}/day.

The order of the experiments was as follows. The cell was filled with the digest (18·3 ml.) by suction through the sintered-glass diaphragm. It was suspended in an outer vessel so that the diaphragm just touched the surface of the water (20 ml.) contained therein. The cell was left at the temperature of the experiment for about 24 hr. in order to establish a uniform diffusion gradient in the sintered glass. The water in the outer vessel was then replaced, and after a period of diffusion the concentration of N which had diffused into it was determined. The liquid in the outer vessel was then replaced by 20 ml. of fresh water, and after a further period of diffusion the concentration in the diffusate was again determined. In this way the concentration outside the cell was usually estimated in three successive periods of diffusion. The cell was then opened and the concentration inside estimated. From the analytical results the concentration inside the cell at the beginning of each diffusion period could be ascertained. The diffusion coefficients were calculated by the formula

$$D = \frac{2{\cdot}303\,V''V'}{Kt\,(V''+V')} \times \log_{10}\frac{C_0''}{C_0''-(1+V'/V'')\,C'},$$

where V', V'' are the volumes of liquid outside and inside the cell, C_0'' the initial concentration in the cell at the beginning of each diffusion run, C' the concentration outside at the end, K is the cell constant and t the time. The measured diffusion coefficient was corrected to 20° by the formula

$$D_{20} = D_x \left(\frac{T_{20}}{T_x}\right)\left(\frac{\eta_x}{\eta_{20}}\right),$$

where η_x, η_{20} are the viscosities of water at $x°$ and 20°. The factor is 1·609 at 3°, 1·557 at 4°, 1·511 at 5°, 1·465 at 6°,

1·302 at 10° and 0·870 at 25°. The molecular weight was determined by the formula

$$\log_{10} D_{20} = -4{\cdot}56 - (1/3)\log_{10} M,$$

which appeared to give the best fit of recorded values of D_{20} for a variety of proteins and other compounds.

Chymotrypsin digests. The solution was usually made by addition of insulin (0·1 g.) to 1·5 ml. of a phosphate buffer (0·5 M, pH 7·6). The pH was then adjusted to about 8·3 by 0·02 N-NaOH (3 ml.) and the volume made up to 15 ml. with water. (This higher pH was chosen because the solubility of insulin is too small at a lower pH; insulin is completely stable in this solution and no change in activity was observed in 5 days at 25°.)

Cystine. The digest fractions were hydrolyzed by refluxing with a mixture of equal volumes of 6 N-HCl and 90% (w/w) formic acid, usually for 19 hr. The cystine content of the hydrolysate was then determined by the method of Kassell & Brand (1938).

RESULTS

Chymotrypsin

The action of chymotrypsin (Fig. 1) is rapid in the early stages, up to the formation of 50% N.P.N. After that it slows down considerably and a long time is necessary to complete the digestion; in fact,

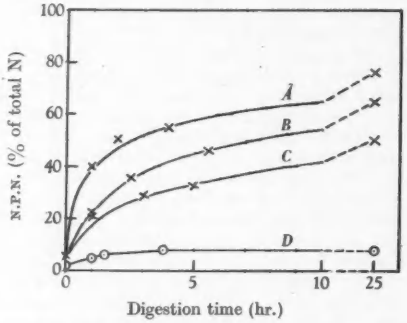

Fig. 1. Action of chymotrypsin and trypsin on insulin. *A*, chymotrypsin, 16×10^{-4} unit/ml.; *B*, chymotrypsin, $3{\cdot}2 \times 10^{-4}$ unit/ml.; *C*, chymotrypsin, $0{\cdot}7 \times 10^{-4}$ unit/ml.; *D*, trypsin, 4×10^{-4} unit/ml.

the N.P.N. only reaches values in the neighbourhood of 100% when more chymotrypsin is added. The digestion process thus consists of a first stage, in which a rapid liberation of about half the total nitrogen takes place, leaving the remainder in a form still precipitable by 0·3 N-trichloroacetic acid. This is followed by a slow digestion of the remaining peptide bonds. The relation between the liberated N.P.N. and amino N in the earlier stages is linear (Fig. 2), the value of the amino N corresponding to 100% N.P.N. being about 22%. This means that the amount of N.P.N. liberated/peptide bond split is approximately the same throughout the reaction, so far as it has been followed. We can estimate this amount in the following way. In the original insulin the free amino N, as determined by the Van Slyke method, is 4·6% of the total (Chibnall, 1946). Taking the N content of pure insulin as 15·54%, a 'sub-molecule' of molecular weight 12,000 (which is the average minimal molecular weight required by all the analytical results) contains a total of 133 atoms of N, of which 4·6% or 6 atoms are in uncombined amino groups. According to Sanger (1945), two of these belong to lysine and the remaining four are terminal α-groups at the free ends of the peptide chains. In the same way, 22% of amino N in the

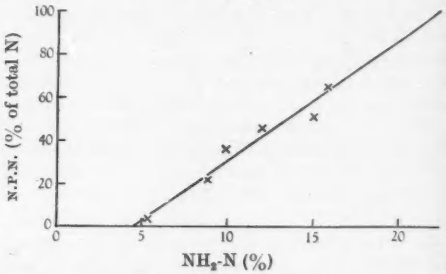

Fig. 2. Action of chymotrypsin on insulin. Relation of amino N to non-protein N.

digest corresponds to 29 amino groups, so that complete digestion with chymotrypsin increases the number of terminal amino groups from 4 to 27. Thus if the breakage of 23 peptide bonds releases 100% of the nitrogen into the 'non-protein' part, assuming linearity as in Fig. 2, each peptide bond broken releases 4·35% of the nitrogen, i.e. 5·8 N atoms.

This estimate was confirmed by a direct determination of the amino N in the trichloroacetic acid filtrate. The solution used was obtained from a digest in which N.P.N. was 58·5%. It was found that the amino N in this filtrate was 22·5%, i.e. *c.* 1 N atom in 4·5 belongs to a free amino group. After complete hydrolysis with 6 N-HCl for 18 hr., the amino N was 79%, i.e. it had increased to 3·5 times its original value, so that the original peptide contains on the average 2·5 peptide bonds for each free amino group, and also 1 atom of N which is not potential amino N. These observations also suggest that the average molecule of N.P.N. is a tetra- or pentapeptide.

Biological activity and splitting. It was found in preliminary physiological tests (see Table 1) that the activity disappeared when about 50% N.P.N. has been formed, i.e. at the end of the rapid reaction, so that the protein-like material precipitated by 0·3 N-trichloroacetic acid, which remains at this point, is biologically inactive. An accurate biological assay of the trichloroacetic acid precipitate was kindly made by Boots Pure Drug Co., Ltd. The dried

Table 1. *Effect of chymotrypsin on the physiological action of insulin*

(The amount injected was equivalent to 0·05 mg. insulin. The results given for pure insulin were the average for 6 rabbits, the remainder obtained by injection of a single rabbit.)

Non-protein nitrogen (%)	0	6	28	33	40·3	50	52	55·2
Average decrease in blood sugar 3 hr. after injection of sample (%)	49·2	55	56	54	51	45	0	0

precipitate from a digest with a N.P.N. value of 58·5 % (see p. 121 for particulars) had an activity of 0·68 unit/mg. (with $P = 0·95$, limits of error 0·56–0·85). On the assumption that the percentage N in the 'protein-like' material was the same as in insulin, the precipitate contained 55 % of 'protein', so that its activity on a weight basis was 5·4 % of that of insulin. It is probable that the protein-like material at this stage of the digestion contained a little unchanged insulin, amounting to *c.* 2 % of the original insulin.

It can be seen (Fig. 3) that it agrees quite closely with the percentage of N precipitated at the isoelectric point,* thus providing a fairly convincing proof that (1) the material precipitated at pH 5·3 is, in fact, unchanged insulin and (2) that trichloroacetic acid precipitates, in addition, material which is not insulin and which does not contribute appreciably to the observed residual activity.

Nature of the split-products. It seemed probable that the initial stage of the reaction consisted of a splitting off of small peptides leaving a compact

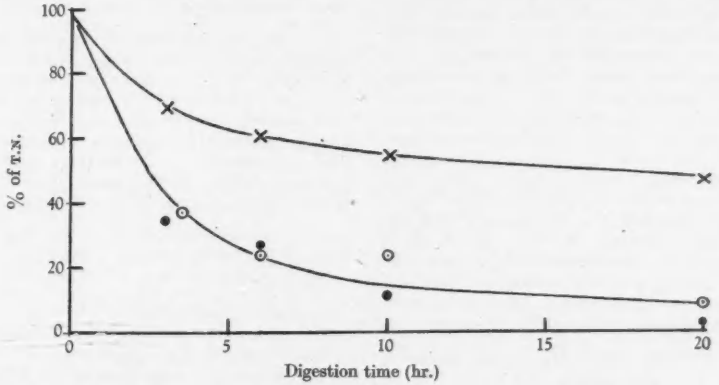

Fig. 3. Action of chymotrypsin on insulin. × Percentage N precipitated by 0·25 N-trichloroacetic acid. ⊙ Percentage N precipitated at the isoelectric point, pH 5·3. ● Percentage of original insulin activity found in trichloroacetic precipitate.

A further determination of the amount of unchanged insulin remaining at various stages of the digestion was made in the following way. Portions (15 ml.) of a chymotrypsin digest were withdrawn at various times and the 'protein' precipitated by trichloroacetic acid at the usual concentration. At the same times samples were added to equal volumes of 0·1 M-acetate buffer of pH 4·7 giving a final pH of 5·3, close to the isoelectric point at which the solubility of insulin is small. The precipitate formed was filtered off after 1 hr. and the N remaining in the filtrate determined. From these data we were able to find the percentage of the original N present in the trichloroacetic precipitate and the percentage of the original N precipitated at pH 5·3 (Fig. 3). The trichloroacetic acid precipitates were dried, weighed and portions assayed for insulin activity by Boots Pure Drug Co. Ltd.

From the results, the total activity of the trichloroacetic acid precipitate was obtained as a percentage of the insulin activity originally present.

protein-like residue. In order to find what proportion of the liberated nitrogen, if any, was derived from free amino-acids, a determination of these latter formed in the first stage was made by the ninhydrin method. The results obtained are given in Table 2. It is evident from these figures that the free amino-acids formed are only a small percentage (about 2 %) of the whole N.P.N. liberated. In the second example shown, the free amino-acid N formed is 1·23 % of the total N; i.e. it is equivalent to 1·6 N atoms per stoicheiometric unit of 12,000.

* The T.N. value of the 10 hr. isoelectric mixture is high because, owing to the time at which this sample was taken, it had to be frozen for a time and filtered about 12 hr. later. After 20 hr. the insulin assay gave a value which was definitely less than that in the isoelectric precipitate, so that in this case the precipitate probably contained material which was not insulin.

Thus between one and two molecules of free amino-acid are formed from the submolecule; i.e. of the 11 peptide bonds broken, one or two may liberate a terminal amino-acid. It is, of course, quite possible that this free amino-acid arises from secondary actions. The greater part of the non-protein fraction must thus consist of peptides with an average chain length, as estimated above, of 4–5 residues.

Table 2. *Products of chymotrypsin digestion*

Digestion period (hr.)	3	30·5
Non-protein nitrogen (% of total N)	31	47·5
Free amino-acid N (% of N.P.N.)	1·8	2·6
Amino N (% of T.N.)	10	12·7
Approx. no. of peptide bonds broken/unit of mol.wt. 12,000	7	11

A further confirmation of this calculation was obtained by determining the molecular weight of the digestion products by the diffusion cell. Insulin (0·5 g.) was digested with chymotrypsin for 17·5 hr. at 25°, the N.P.N. at the end of this period being 58·5%. Trichloroacetic acid was then added to a final concentration of 4%, and after 0·5 hr. the precipitate was filtered off (dry wt. 340 mg.). The filtrate deposited some further precipitate on standing which was removed and the pH was then adjusted to 4·4. This filtrate was then put in the diffusion cell, and after preliminary contact with water to set up the diffusion layer, the amount diffusing out in three successive portions of initially pure water was determined. From these results, and the final concentration in the diffusion cell, the diffusion coefficients were determined and corrected to 20° (Table 3). The values found correspond to a molecular weight of 800±200, which is in good agreement with the figure estimated above. It will be observed that the values of D diminish in successive determinations; this may be due to material of lower molecular weight diffusing out first.

Table 3. *Diffusion coefficient of trichloroacetic acid filtrate*

Temp. (°)	Diffusion time (hr.)	Concentration in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$	$D_{20} \times 10^6$
5	66·5	0·013	2·05	3·26
5	76·5	0·013	1·87	2·97
4	90	0·013	1·68	2·77

Final conc. in diffusion cell (18·3 ml.) was 0·268 mg. N/ml. Mean D_{20} sec.$^{-1} = 3{\cdot}0 \pm 0{\cdot}3 \times 10^{-6}$.

The trichloroacetic acid precipitate was now dissolved (4 mg./ml.) in 0·02N-NaOH and the pH adjusted to 8·0. This liquid was put in the diffusion cell and after a preliminary contact with water for 24 hr. the amounts of N diffusing out in successive times were determined (Table 4).

Trichloroacetic acid thus divides the digest into a soluble part, consisting of comparatively small peptides of mol.wt. *c.* 800 and an insoluble part of average mol.wt. *c.* 4000. The former makes up about half the molecule. The insoluble part has a molecular weight which agrees quite well with that of a submolecule of mol.wt. 12,000 from which rather more than half the residues (i.e. about 60) have been removed. The action of chymotrypsin therefore appears to be a removal of the outer parts of the insulin molecule leaving a fairly massive nucleus. This process is complete at the end of the first stage of the digestion (50% N.P.N.). In the later slow stage the massive nucleus is decomposed into smaller non-precipitable fragments.

It is surprising that trichloroacetic acid can precipitate molecules with molecular weight as low as *c.* 5000, well below the order of magnitude usually considered as proteins. This finding must cause some revision in our ideas of what constitutes the 'protein' and 'non-protein' parts of a digest. In view of this we determined the effect of varying the trichloroacetic acid concentration on the amount precipitated, with the following results: 1 ml. digest was added to 5 ml. of trichloroacetic acid of concentration shown:

Conc. of trichloroacetic acid (N)	0·15	0·3	0·6	1·0
Percentage of T.N. remaining as N.P.N.	74	51	48	47

There is thus no significant increase in the proportion of N precipitated at concentrations above that usually employed (0·3N). When 1 ml. of the digest was added to 9 ml. of acid tungstate, prepared according to Van Slyke & Hawkins (1928), an even greater percentage, viz. 61·6% of T.N., was precipitated.

When added to an acidified chymotrypsin digest, pepsin produces little further splitting. For example, a chymotrypsin digest which contained 14·2% amino N, was acidified to pH 2 and 9×10^{-4} pepsin unit/ml. was added. After 24 hr. the amino N was 15·0%.

Distribution of cystine and of amino N. Since treatment of the 50% digest with trichloroacetic

Table 4. *Diffusion coefficient of trichloroacetic acid precipitate*

	Temp. (°)	Diffusion time (hr.)	Concentration in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$	$D_{20} \times 10^6$
Series 1	4	65·5	0·007	1·02	1·68
	4	96	0·006	1·24	2·04
	Final conc. in diffusion cell, 0·223 mg. N/ml.				
Series 2	3	96	0·0066	0·93	1·58
	3·5	96	0·0063	0·91	1·54
	5	123	0·010	1·18	1·88
	Final conc. in diffusion cell, 0·213 mg. N/ml.				

Mean D_{20} sec.$^{-1} = 1{\cdot}74 \pm 0{\cdot}2 \times 10^{-6}$, corresponding to a mol.wt. of about 4000±1500.

acid divides it into two fractions, the distribution of the more important amino-acids between them is of interest. We report below two preliminary experiments on the distribution of cystine. They are not entirely satisfactory, as only about 90% of the total cystine is accounted for, but this loss can hardly affect the main point which emerges.

Exp. 1. Recrystallized insulin (0·50 g.) in 90 ml. solution, pH 8·2, was incubated with chymotrypsin (3 mg. protein N). After 17·5 hr., N.P.N. was 58·5%. To 84 ml. of digest was added trichloroacetic acid to 0·24 N; the precipitate, which was filtered off after 30 min., amounted to 340 mg. Both precipitate and filtrate were hydrolyzed with the HCl-formic acid mixture for 19 hr. and cystine contents of the hydrolysates were determined (Table 5).

Table 5. *Distribution of cystine in the soluble and insoluble fractions of a partial digest of insulin*

(The figures of T.N. and cystine N are expressed in mg./10 ml. of original digest.)

Exp. 1:	Total nitrogen (mg.)	Cystine N (mg.)	$\frac{\text{Cystine N} \times 100}{\text{Total N}}$
Digest	8·05	—	—
Precipitate	3·40	0·504	14·7
Filtrate	4·67	0·114	2·5
Exp. 2:			
Whole digest	7·67	0·618	8·07
Precipitate	4·24	0·476	11·23
Filtrate	3·38	0·151	4·47

In the original solution the N.P.N. was 6% of the whole, so that the original protein N was 63·5 mg. This should contain 5·95 mg. of cystine N (9·36%), of which 5·19 mg. (i.e. 87%) were accounted for.

Exp. 2. In this case the final concentration of trichloroacetic acid was 0·25 N; the precipitate which formed was filtered off but not dried. It was prepared after 33·7 hr. digestion (with lower chymotrypsin concentration) when N.P.N. was 44·1%. The precipitate was dissolved in a little N-KOH and diluted. Digestion with the HCl/formic acid mixture was carried out for 8 hr. The results are given in Table 5. In this experiment the sum of the cystine concentrations in the two fractions agrees with that determined for the original digest, but both are only 90% of that expected for the total cystine of the insulin.

In both experiments the proportion of cystine in the precipitate is considerably greater than that in the filtrate, and the ratio is greater for the digest which has been digested further.

The proportion of amino N in the precipitate (55% of T.N.) was found to be 8·7%, which means an average chain length in the precipitated material, assuming the same average composition as insulin, of 9–10. This also fits in with the general picture that the enzyme has removed about 10–12 peptide fragments averaging 6 residues from the original submolecule of 106 residues in 4 chains, leaving about 40 residues in 4 chains.

Trypsin

Contrary to statements in the literature, trypsin itself had a very slight action on insulin. When 24×10^{-4} unit of trypsin was added to 5 ml. of a solution containing 17 mg. of insulin/ml. at pH 8·2, the increase of N.P.N. was only 4% in 24 hr. at 25° (Fig. 1). Insulin was recovered from a solution containing 5·5 mg. insulin and $1·4 \times 10^{-4}$ unit of trypsin/ml. after standing at 2·5 hr. at 25° and 16 hr. at 5°. From 14 ml. of this solution which originally contained 77 mg. insulin, 75·4 mg. of insulin were crystallized. An accurate assay of this material was kindly made for us by Boots Pure Drug Co. Ltd., who found that it had 4·03% moisture and an activity of 22·96 units/mg. corresponding to 23·93 units/mg. of dried crystals. It was therefore indistinguishable from the purest insulin. A much stronger trypsin solution (1 ml.; 0·73 mg. protein N, 800×10^{-4} unit/ml.) was added to 15 ml. of an insulin solution at pH 8·4; this produced about 10% N.P.N. in 4 hr. and 50% N.P.N. in 24 hr. Such an action might be produced by the presence of approximately 0·5% of chymotrypsin in the trypsin. The trypsin was therefore tested by the milk-clotting method as used by Kunitz (1935) and Herriott (1938) standardized with pure chymotrypsin; the activity corresponded to the presence of 0·4% chymotrypsin. It was therefore concluded that the slight action observed was due to chymotrypsin present and that trypsin itself has no significant action at pH 8·2. In order to find if any action of trypsin occurred at a lower pH, a solution was made containing 1 mg. of insulin/ml. at pH 7·0. Trypsin produced no appreciable change of amino N and no decrease in biological activity.

Trypsin can, however, hydrolyze further the products after digestion of chymotrypsin, if present in considerable amount. A chymotrypsin digest to which trypsin was added at a concentration of 0·05 mg./ml. changed very little in 19 hr.; but at a concentration of 0·5 mg./ml. the amino N rose overnight from 15·8 to 20·8%, while the increase in the same time without trypsin was only 3%. It follows that the rate of digestion of insulin by trypsin and chymotrypsin together will be greater than the sum of their separate effects.

SUMMARY

1. The action of crystalline trypsin and chymotrypsin on insulin has been studied. Crystalline trypsin has no appreciable effect on insulin, but is capable of digesting the products formed after chymotrypsin action.

2. The action of chymotrypsin occurs in two stages. The first stage consists of a rapid action leading to the formation of about 50% non-protein

N as determined by precipitation with 0·25N-trichloroacetic acid. In the second stage, the non-protein N rises slowly to the 100% value. The biological action disappears at about the end of the first stage. Diffusion experiments showed that the 'protein-like' material left here had a molecular weight of only 4000, whilst that of the soluble fraction was about 800. The initial action of chymotrypsin thus involves the cleavage of about 10 or 11 peptides containing on the average 5 or 6 residues from each submolecule, leaving a fairly massive core. The latter is broken down in the second stage of the action.

3. The amino-N content of the 'core' indicates a probable chain length of 10–12 residues. The greater part of the cystine (from 75 to 85%) is in the core.

One of us (J.A.V.B.) holds the Courtauld Research Fellowship in this Institute. Our thanks are due to Messrs Courtauld Ltd., in addition, for a grant in aid of this investigation; also to the Imperial Chemical Industries for financial assistance, to Sir J. Drummond, F.R.S., Research Director of the Boots Pure Drug Co. Ltd., for facilities in the preparation of some of the enzymes and for arranging assays of insulin, and to Drs J. H. Northrop and M. Kunitz for a sample of crystalline trypsinogen.

REFERENCES

Behrens, O. K. & Bergmann, M. (1939). *J. biol. Chem.* **129**, 587.
Chibnall, A. C. (1946). *J. int. Soc. Leath. Chem.* **30**, 1.
Consden, R., Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1946). *Biochem. J.* **40**, xliii.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1941). *Biochem. J.* **35**, 1369.
Gordon, A. H., Martin, A. J. P. & Synge, R. L. M. (1943). *Biochem. J.* **37**, 92.
Herriott, R. M. (1938). *J. gen. Physiol.* **21**, 501.
Hill, D. W. & Howitt, F. O. (1936). *Insulin, its Production, Purification and Physiological action.* London: Hutchinson.
Jensen, H. F. (1938). *Insulin, its Chemistry and Physiology.* New York: The Commonwealth Fund.
Kassell, B. & Brand, E. (1938). *J. biol. Chem.* **125**, 115.
Kunitz, M. (1935). *J. gen. Physiol.* **18**, 459.
Kunitz, M. & Northrop, J. H. (1935). *J. gen. Physiol.* **18**, 433.
Kunitz, M. & Northrop, J. H. (1936). *J. gen. Physiol.* **19**, 991.
McDonald, M. R. & Kunitz, M. (1946). *J. gen. Physiol.* **29**, 155.
Mehl, J. W. & Schmidt, C. L. A. (1937). *Univ. Calif. Publ. Physiol.* **8**, 165.
Northrop, J. H. & Kunitz, M. (1932). *J. gen. Physiol.* **16**, 267, 295.
Sanger, F. (1945). *Biochem. J.* **39**, 507.
Synge, R. L. M. (1944). *Biochem. J.* **38**, 285.
Synge, R. L. M. (1945). *Biochem. J.* **39**, 363.
Van Slyke, D. D., Dillon, R. T., MacFadyen, D. A. & Hamilton, P. (1941). *J. biol. Chem.* **141**, 627, 671.
Van Slyke, D. D. & Hawkins, J. A. (1928). *J. biol. Chem.* **79**, 739.

The Action of Pepsin on Insulin and the Plastein Question

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The course of the action of pepsin on insulin, and the products of the digestion, are quite different from those observed with chymotrypsin. On the whole, the breakdown produced by pepsin is less extensive than that observed with chymotrypsin. The most interesting feature of the action is the diminution, under certain conditions, of the apparent 'non-protein' nitrogen in the digests. This phenomenon appeared to be similar to the process of plastein formation, which has been known for a considerable time. Wasteneys & Borsook (1924, 1925)* reported that when a peptic digest of egg albumin is concentrated, and then incubated with more pepsin at pH 4 (at which pH the proteolytic action of pepsin is slight), the 'non-protein' nitrogen, determined as the nitrogen soluble in the presence of 2% trichloroacetic acid, is diminished. They regarded this as evidence of the synthesis of protein-like material, 'plastein', by the action of pepsin at pH 4, citing as contributory evidence the fact that the plastein, when filtered off, contained an appreciably smaller proportion of amino N than the original digest. They studied the effect of such variables as pH, temperature and concentration, and reported that when boiled pepsin was added instead of the active enzyme, no synthesis was observed. The matter was re-examined by Folley (1932), who concluded that there was no proof of resynthesis and found that plastein formed from egg albumin had a comparatively low molecular weight.

* Wasteneys & Borsook give references to earlier observations of this kind.

Similar observations were made with a peptic digest of insulin by Fisher & Scott (1933), who used the decrease of material soluble in 3·3 % trichloroacetic acid as the criterion of plastein formation during incubation at pH 4. Haddock & Thomas (1942) found that trypsin had a similar action at pH 5·7 on the peptic digest of insulin, as had papain on both peptic and papain digests. The circumstances under which insoluble material is formed in insulin digests have been investigated in some detail in this paper.

EXPERIMENTAL

The pepsin was a crystalline product presented by Parke, Davis and Co. It was kept at −10° as a 2% stock solution in 60% glycerol adjusted to pH 5 with sodium acetate and acetic acid; dilutions were prepared as required. The activity as determined by the method of Anson (1938) was 0·54 unit/ml.

Non-protein nitrogen (N.P.N.). At first the digest was added to boiling trichloroacetic acid (0·3N) in order to inactivate the pepsin, but this was unsatisfactory owing to the uncertain amount of evaporation, and it was found that addition of trichloroacetic acid at the same strength at room temperature gave nearly the same results (Table 1).

The trichloroacetic acid mixture was not allowed to stand too long before filtration, as a slow precipitation of insoluble material might occur. Certain of the pepsin digests which gave no immediate precipitate with trichloroacetic acid gave one after standing for 1–2 hr. The time of standing was standardized at 30 min., which in certain cases of complete digestion gives too low a value for N.P.N.

Table 1. *The determination of non-protein* N (N.P.N.) *in an insulin digest by trichloroacetic acid* (T.C.A.) *at room temperature and at the boiling point respectively*

(Insulin (60 mg.) in 12 ml. of 0·05N-HCl; 1 ml. of 1/100 dilution of stock pepsin.)

Time after adding pepsin (hr.)	N.P.N. (mg./ml.) T.C.A. at room temp.	T.C.A. (boiling)
1	0·21	0·21
2·5	0·31	0·29
5	0·62	0·53

Peptic digests. The solution used usually contained 5 mg. of insulin dissolved in 1 ml. of 0·05N-HCl, to which was added the desired amount of pepsin, usually at a dilution of 1 : 100 of the stock solution.

RESULTS

Extent of the action. The N.P.N. rises smoothly and quickly to values close to 100%, while the amino N reaches and remains at a constant value (with small pepsin concentrations) of about 12–13% (Fig. 1). This may be followed by a very slow action of pepsin on the primary split-products, the amino N reaching 13–14% in 24 hr.; with concentrations of pepsin 5 or 10 times as great the amino N reached 16% in 2 days. The 13% stage is, however, fairly definite and easily reproduced. During this initial action there is a linear relation between the N.P.N. and amino N (Fig. 2), which indicates that the amount of N.P.N. liberated/peptide bond split is the same throughout the action of the enzyme. Thus the hydrolysis of the peptides initially formed is relatively slow.

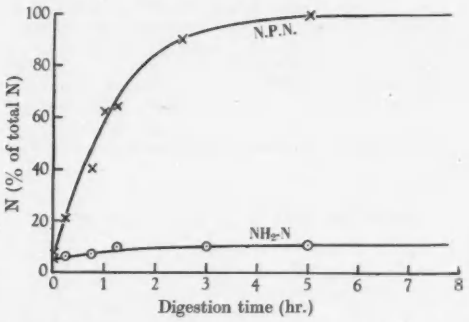

Fig. 1. Action of pepsin on insulin at pH 2. Change of amino N and N.P.N. with time. Pepsin units/ml. of digest, 3×10^{-4}.

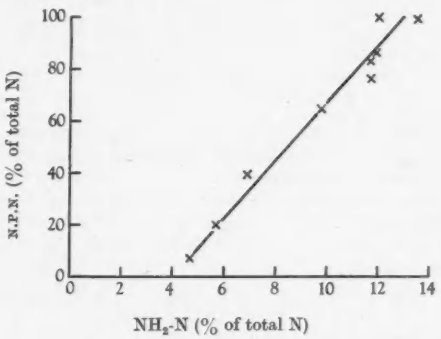

Fig. 2. Action of pepsin on insulin. Relation between amino N and N.P.N.

By the same method of calculation as used in the previous paper (p. 118), it was found that 13% of amino N in the digest corresponds to 17 amino groups, so that the digestion has increased the number of terminal amino groups from 4 to 15. The average peptide chain in the digest has a molecular weight of 12,000/15 = 800 and thus contains about 7 amino-acid residues.

This gives, of course, no information about the uniformity of the products. The amount of uncombined α-amino-acids in the digest as determined by the Van Slyke ninhydrin method was 0·7% of the

total N. It follows from this that in the digestion product from one unit of 106 residues (mol.wt. 12,000), less than one is present as uncombined amino-acid, i.e. of the ten peptide bonds broken not more than one belongs to a terminal amino-acid. Indeed, the small amount found may be the result of a secondary reaction.

Diffusion coefficient. A peptic digest was prepared by mixing 200 mg. insulin in 40 ml. of 0·05 N-HCl with 3 ml. of a 1 : 200 dilution of the stock pepsin. After standing overnight at 25° it gave no immediate precipitate with trichloroacetic acid. To destroy the pepsin, the digest was now neutralized to pH 7·2 and heated to 80° for 3 min. The precipitate thus formed was filtered off and when dried amounted to 42 mg., i.e. to about 20% of the original insulin. The nature of this 'heat precipitate' is discussed below. Diffusion coefficients were determined on the filtrate (Table 2).

Table 2. *Diffusion coefficient of digest filtrate*

	Temp. (°)	Diffusion time (hr.)	Conc. in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$	$D_{20} \times 10^6$
Series 1	25	23·5	0·0075	2·21	1·92
	Final conc. inside cell, 0·453 mg. N/ml.				
Series 2	10	49	0·009	1·52	2·05
	5	47	0·006	1·07	1·71
	6	48	0·007	1·32	2·02
	Final conc. inside cell, 0·359 mg. N/ml.				

Average D_{20} sec.$^{-1} = 1{\cdot}92 \times 10^{-6}$.

The average molecular weight of a peptide corresponding to the diffusion coefficient (Table 2) is 3000 ± 1000, which is significantly higher than that of the average peptide chain in the digest. It follows that the diffusing molecule must contain on the average about 4 peptide chains. These might be held together in various ways, but the disulphide bonds, of which there are only 1·5 in a peptide of mol.wt. 3000, are insufficient by themselves for this purpose.

The plastein question

In the course of our experiments we noticed on one occasion that the N.P.N., which had been nearly 100% after digestion for 5 hr., apparently diminished to approximately 50% after 24 hr. without any diminution of the amino N. This was found to be due to the fact that in the latter determination the trichloroacetic mixture had stood for an abnormally long time before being filtered. The following experiment shows the nature of this action. A series of tubes was made up containing 1 ml. of a fresh peptic digest and 5 ml. of 0·3 N-trichloroacetic acid and the mixtures were allowed to stand at room temperature. Precipitate was first observed after 1 hr. At various intervals the precipitates in the separate tubes were filtered off and the N.P.N. was determined; the results are given in Table 3.

Table 3. *Decrease of non-protein* N (N.P.N.) *in a peptic digest of insulin after various periods*

Time (hr.)	N.P.N. (mg./ml.)	N.P.N. (% of total N)
1·5	0·57	88·0
3	0·36	55·4
7	0·34	52·5
24	0·29	44·7

The precipitation of some material by trichloroacetic acid can hardly be due to pepsin, since the latter is inactive in such a solution. However, it appeared possible that it was caused by a hydrolytic action of the acid.

It will be recalled (p. 124) that, when a peptic digest is neutralized and heated to 80° for a few minutes, a precipitate is formed which may amount to 20% or more of the original insulin and continues to form in the filtrate at a slower rate at lower temperatures. Indeed, when the filtrate was frozen and stored at −10°, it was usually found that some insoluble matter was present on thawing. It therefore appeared that the peptic digest undergoes some change on being kept at certain acidities, which results in the formation of material which is both insoluble by itself

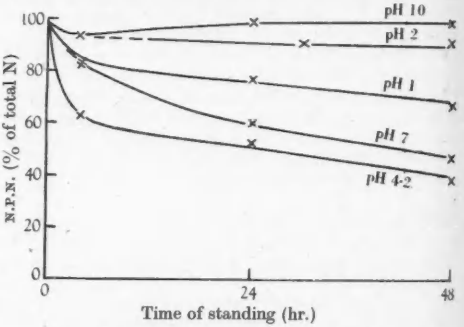

Fig. 3. Changes in peptic digest at 25° with pH.

and in the presence of trichloroacetic acid. In order to find under what circumstances it is formed, a peptic digest was made which gave no immediate precipitate with trichloroacetic acid. The pH of measured samples of this digest was adjusted to different values. After standing for various times at 25°, samples were added to 0·3 N-trichloroacetic acid, the precipitate formed after 30 min. was filtered off, and the N in the filtrate determined (Fig. 3). Except in the alkaline solution a considerable drop in N.P.N. occurred. The percentage of amino N in the whole mixture was determined by the Van Slyke method (Table 4). There is a slight

Table 4. *Change of amino* N *and non-protein* N *with time in peptic digest*

		After standing for 24 hr. at pH			
	Original	1	4·2	7·0	*c.* 10
Amino N (% of total N)	13·0	13·0	12·7	13·0	13·7
N.P.N. (% of total N)	100	80·8	54·3	60	100

diminution of the amino N in the three solutions in which N.P.N. has diminished, amounting at most to about 1%. This would correspond to the resynthesis of one peptide bond out of the eleven broken; but it is doubtful whether the determinations are sufficiently accurate, since they are made with a mixture of solid and liquid. It is quite possible that under these circumstances small variations would occur with constant reaction time according to the amount of solid. At any rate, the figures do not support the suggestion that any considerable synthesis of peptide bonds, corresponding to the amount of solid formed, has occurred.

In order to find if the changes observed are due to the pepsin still remaining in the solutions, a further series of experiments was made in which the peptic digest was heated to 60° for 5 min. at pH 7·8 before adjusting the pH to its final value. It was shown that the activity of the pepsin was completely destroyed by this treatment. The pH was then adjusted by the addition of HCl to 2·0, 4·0 and 7·0, and after standing for various times at 25°, samples were taken for analysis. The results are shown in Fig. 4.

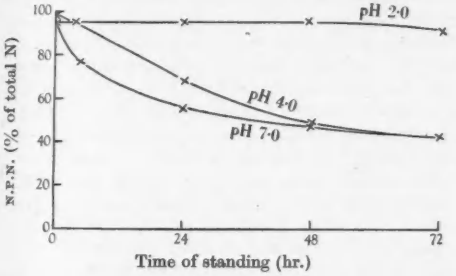

Fig. 4. Changes in peptic digest at 25° with pH after inactivation of the pepsin.

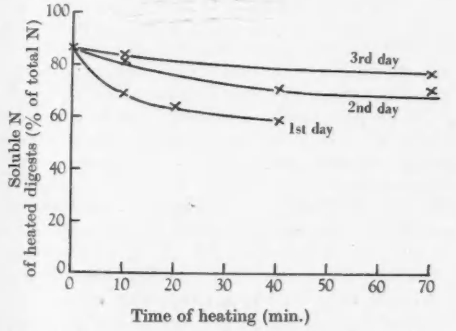

Fig. 5. Effect of age of peptic digest on amount of precipitate formed on heating.

At pH 4·0 and 7·0 the N.P.N. diminishes steadily, reaching about 50% after 2 days, while there is very little change with time when the pH has been restored to its original value. This experiment shows that the presence of active pepsin has nothing to do with the drop of the N.P.N. observed in our experiments.

In the course of this work it was noticed that the quantity of precipitate obtained on heating appeared to be less with an old digest than with a fresh one. To test this a peptic digest was prepared in the usual way. After 18 hr. a portion was neutralized to pH 7 and heated for various times. The precipitates formed were removed by filtration and the amounts of N remaining in the filtrates were determined. The same process was repeated with digests 42 and 66 hr. old (Fig. 5). It can be seen that the amount of precipitate obtained diminishes with the age of the digest.

In this experiment the amount of N precipitated by trichloroacetic acid was also determined (Table 5). In nearly every case the amount agrees with or is only slightly less than the amount precipitated by heating, thus confirming the suggestion that the material precipitated by heating is identical with that precipitated by trichloroacetic acid.

Table 5. *Insoluble matter formed on heating digest*

Age of digest (hr.)	Time of heating (min.)	Percentage of N not precipitated by heat	Percentage of N not precipitated by T.C.A.
18	10	69	69
	20	64	61
	40	59	56
42	10	84	79
	40	71	75
	70	71	65
66	10	85	87
	70	77	74

Properties of the precipitate and filtrate. The precipitate used was obtained from 500 mg. insulin at the usual concentration, digested with 2 ml. of a 1 : 50 dilution of the stock solution of pepsin for 17 hr. The digest was neutralized and heated to 75° for 10 min. The dried precipitate (6A) amounted to 88 mg. After being frozen for 1 week a further 83 mg. of precipitate (6B) was removed. The total precipitate, allowing for filtrate used in tests, was about 40% of the insulin taken.

The precipitate 6A dissolved slowly (1 mg./ml.) in 0·05 N-HCl. Its total N amounted to 15·4% of the dry weight. The precipitate is complex. The following amino-acids have been identified in an acid hydrolysate in a preliminary analysis by paper chromatography: aspartic acid, cystine, tyrosine, glutamic acid, glycine, alanine, leucine. There are almost certainly other amino-acids present, as several spots were not identified. The amino N as a percentage of total N in the precipitate 6A, as determined by Van Slyke's method, was 8·2%; in 6B, 8·7%, and in the filtrate from which both 6A and 6B had been removed, 16·2%.

It can be seen that the process of heating divides the digest into two parts containing respectively a smaller and greater proportion of amino N than the original digest. It is impossible to determine the average chain lengths in the fractions without knowing the distribution of the lysine, etc., but on the assumption that the average composition of each is similar to insulin, the precipitates have a peptide chain length of about 10–12 residues, while the filtrate has an average chain length of about 6. The molecular weight of precipitate 6A was determined in the diffusion cell in the usual way (Table 6).

Table 6. *Diffusion coefficient of heat precipitate*

Temp. (°)	Diffusion time (hr.)	Conc. in 20 ml. diffusate (mg. N/ml.)	$D \times 10^6$
25	41	0·0025	1·35
25	72	0·005	1·58
25	72	0·004	1·32

Original conc. in cell, 0·144 mg. N/ml.

Mean: D_{25} sec.$^{-1} = 1{\cdot}42$, $D_{20} = 1{\cdot}24 \pm 0{\cdot}2 \times 10^{-6}$; mol.wt. = 11,000 ± 4000.

A certain amount of the high molecular weight precipitate had been removed from the digest used in the previous molecular-weight determination (Table 2). In view of this, it was desirable to determine the molecular weight in a filtrate from which as much as possible of the heat precipitate had been removed. This was done with filtrate 6.

Table 7. *Diffusion coefficient of filtrate*

Temp. (°)	Diffusion time (hr.)	Conc. in diffusate (20 ml.)	$D \times 10^6$
25	48	0·011	2·50
25	48	0·0108	2·55

Final conc. in cell, 0·273 mg. N/ml.

Mean: D_{25} sec.$^{-1} = 2{\cdot}52$, $D_{20} = 2{\cdot}18 \times 10^{-6}$; mol.wt. = 2000 ± 800.

It was heated at 80° for 30 min. before use and filtered, but very little additional precipitate formed. The filtrate was introduced into the diffusion cell, and the molecular weight was found to be somewhat lower than the previously determined value, but still clearly higher than the average peptide chain length in the filtrate.

Table 8. *Summary of Results*

	% of total N as			
	Amino N	Cystine N	Chain length	Mol. wt. (order of)
Precipitate (A)	8·2	12·7	10	11,000
(40%) (B)	8·7	13·2		(I4,000)
Filtrate (60%)	16·2	7·9	5	2,000 (I 800)

DISCUSSION

The idea that a resynthesis of protein-like material takes place in the presence of pepsin under certain conditions was based on the observations that: (1) the proportion of nitrogen not precipitated by trichloroacetic acid (N.P.N.) is decreased, (2) the proportion of amino N in the precipitate is lower than that in the digest, and (3) that the phenomenon was not observed when boiled pepsin was added to the concentrated digests. In our experiments the formation of a precipitate from soluble pepsin digests occurred under circumstances in which the pepsin had been definitely inactivated. The pH and temperature are the main factors conditioning its formation. It is not produced at or about pH 2, but it is formed both in more acid and more alkaline solutions. The insoluble material appears to be identical with that precipitated by trichloroacetic acid. The fact that the proportion of amino N in the precipitate is less than in the digest as a whole cannot be cited as evidence of any resynthesis of peptide bonds, but merely means that the average chain length of the peptides in the precipitate is greater than that in the whole digest.

The general picture of the peptic digestion which we obtain is the breakage of about 11 peptide bonds in each submolecule of weight 12,000, giving a product which is initially soluble in the solvent in which it is formed and in the presence of trichloroacetic acid. Later, insoluble material is precipitated from this digest over a considerable range of pH and also in the presence of trichloroacetic acid. What is the nature of the reaction involved? The process is accelerated by heating, but occurs slowly even in the frozen state. It might be a hydrolytic reaction giving rise to an insoluble peptide, but there is no evidence that peptide bonds are split and moreover the effect of acidity does not support this explanation. We have also shown that there is probably no significant decrease of amino N during the formation of the precipitate, so that an appreciable resynthesis of peptide bonds is also ruled out.

The phenomenon seems to resemble that of protein denaturation. When insulin solutions are heated, insoluble denatured protein is formed by changes which do not involve the disruption of peptide bonds, but apparently merely a 'disordering' of the peptide chains which brings more hydrophobic material in contact with water and thus decreases solubility. It may be supposed that some of the larger protein fragments produced by pepsin may retain the structural configuration of the original insulin and the particular arrangement of the component groups, presumably with hydrophilic groups pointing outwards and hydrophobic groups inwards. Heating and addition of trichloroacetic acid might then produce a rearrangement or 'disordering' of these larger fragments, similar to that caused by heat on insulin itself, but occurring at a lower temperature. When the digest is exposed to pepsin for a longer time, the amount of insoluble substance which can be formed diminishes. This might be due to the original fragments being further split to smaller molecules which are no longer capable of this change. Table 8 summarizes the characteristics of the material in the precipitate and filtrate, so far as they have been determined. The molecular weight of the precipitated material is of the same order as that of the submolecule of insulin. It must therefore either be a more or less intact submolecule, or must be made up of portions of more than one submolecule. In the former case the percentage of amino N would be the same as insulin. The simplest interpretation of all the facts is that

pepsin, like chymotrypsin, splits off terminal groups. The length of the peptide chains is not much greater than in the case of chymotrypsin, but they are aggregated in some way to give a diffusing molecule containing about three average peptide chains.

The residue of the insulin molecule is soluble when first formed, but undergoes changes on heating, etc., over a wide range of pH, except near pH 2. The residual molecule probably comes from more than one submolecule and may be derived from one half or more of the whole insulin molecule. The cystine is approximately equally divided between precipitated material and the soluble peptides. This possibly means that pepsin eats more deeply into the insulin 'core' than chymotrypsin in the initial stage of its action. Finally, we may observe that the plastein from egg albumin was shown by Folley (1932) to have a comparatively low molecular weight (<1000). It is likely that the mode of formation of this material, which requires more pepsin at pH 4, differs from that of the material from insulin, and the two phenomena are really dissimilar.

SUMMARY

1. The action of pepsin on insulin at pH 2 has been studied. Insulin is quickly and completely transformed into products which are not initially precipitated by trichloroacetic acid.

2. On standing at various pH values the digest undergoes changes whereby material is formed which is insoluble both in the presence of trichloroacetic acid and in the chosen buffer. This precipitate is similar to the so-called 'plastein', the formation of which has been observed in peptic and other digests.

3. The conditions of formation of the precipitate have been studied. No evidence has been obtained that it requires active pepsin for its formation, or that any appreciable resynthesis of peptide bonds occurs. It is suggested that the larger protein fragments originally formed retain much of the order of the protein, and may suffer changes similar to those which occur in protein denaturation.

4. The molecular weight of the precipitated material is of the order of 12,000 (chain length 10–12 residues), while that of the soluble split-products is *c.* 2000 (chain length about 5 residues). In both cases the diffusing molecule must be an aggregate containing several peptide chains.

One of us (J.A.V.B.) holds the Courtauld Research Fellowship in this Institute. Our thanks are also due to Messrs Courtauld Ltd. for a grant in aid of this investigation, and to the Imperial Chemical Industries for financial assistance. Sir J. Drummond, F.R.S., Research Director of Messrs Boots Pure Drug Co. Ltd., has very kindly supplied certain materials. We have also to thank Messrs Parke, Davis and Co. Ltd. for the supply of crystalline pepsin.

REFERENCES

Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Fisher, A. M. & Scott, D. M. (1933). *J. gen. Physiol.* **16**, 741.
Folley, S. J. (1932). *Biochem. J.* **26**, 99.
Haddock, J. N. & Thomas, L. E. (1942). *J. biol. Chem.* **144**, 691.
Wasteneys, H. & Borsook, H. (1924). *J. biol. Chem.* **62**, 15, 633.
Wasteneys, H. & Borsook, H. (1925). *J. biol. Chem.* **63**, 563, 575.

The Biochemistry of the Gas Gangrene Toxins

3. DEVELOPMENT OF A MEDIUM SUITABLE FOR THE LARGE SCALE PRODUCTION OF THE TOXINS OF *CLOSTRIDIUM WELCHII* TYPE A

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In a previous paper (van Heyningen, 1941*b*) it was reported that, in order to obtain the toxins of *Clostridium welchii* type A, the organism was grown on a solution of commercial peptone supplemented with a protein-free extract of fresh muscle (Macfarlane & Knight, 1941). This medium has a low protein content which is desirable when the toxins are to be purified. Later it was necessary to produce the toxins on a scale of several thousand litres of culture filtrate. It was then found that the commercial peptone did not regularly produce good yields of toxin and accordingly another medium was developed. This medium consists essentially of papain digest of horse muscle pre-treated with alkali. In this work the main interest was in the production of the α-toxin (lecithinase).

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METHODS

(1) *Estimation of toxins*

α-Toxin was determined by measuring the combining power with specific antitoxin. 1 ml. quantities of a dilution of antitoxin containing 1 unit/ml. were pipetted into a number of tubes. Decreasing amounts of the toxin preparation, differing from each other by 10%, were added, and after standing for 30 min., 0·5 ml. of an egg-yolk solution (Macfarlane, Oakley & Anderson, 1941) was added. The tubes were placed in a water bath at 38°, and after 1 hr. readings were taken. The tube which contained the minimal amount of toxin free to react and cause turbidity contained one Le unit of lecithinase. The Le unit is that amount of toxin which in the presence of 1 unit of antitoxin contains a trace of free toxin; unless the toxin is considerably toxoided it is slightly larger than the Lf (flocculating) unit which is exactly neutralized by 1 unit of antitoxin. In fresh culture filtrates 1 Le is generally equivalent to about 20 egg units (E.U., van Heyningen, 1941*a*) or to about 60 average lethal doses (mouse, intravenous); these relationships are of course dependent upon the degree of toxoiding of the toxin. *θ*-Haemolysin was estimated in an analogous manner, with sheep red blood cells as substrate. The diluent was M/15-phosphate buffer, pH 7·4, 10^{-3}M with respect to thiolacetic acid, and containing enough α-antitoxin effectively to inhibit the α-toxin which is also haemolytic.

Collagenase. The κ-toxin (collagenase) antitoxin combining power of the filtrates was estimated by the method of Oakley, Warrack & van Heyningen (1946).

(2) *Other estimations*

Total N was determined by micro-Kjeldahl, amino N by the copper method of Pope & Stevens (1939). Iron was determined colorimetrically with αα′-dipyridyl. pH was determined electrometrically with a dipping glass electrode.

EXPERIMENTAL

A number of enzyme digests of fresh and old (1 month in a refrigerator) horse muscle and of casein were prepared, with hog stomach and commercial pepsin, pancreatic extract and commercial trypsin, and commercial papain as the sources of proteolytic enzymes. Variations were made in the time and temperature of digestion and in the ratios of non-dialyzable and amino N to total N. Supplements such as protein-free muscle extract and yeast extract were added to these digests, but in no case was a satisfactory yield of toxin obtained from cultures grown on media made from the digests, although the organism always grew profusely. Occasionally a non-reproducible yield of about 1 Le/ml. was obtained. Then it was discovered that if the meat was treated with alkali before digestion, and if the digestion was carried out with the meat in high concentration, a medium could be made upon which satisfactory yields of toxin (3–5 Le/ml.) could be obtained.

Effect of alkali on pH *of meat.* 2 kg. quantities of finely minced and defatted meat were mixed with 1 l. quantities of distilled water to which had been added various volumes of 2N-NaOH ranging from 60 to 140 ml. The mixing was done in a Hobart Dough Mixer at high speed. It was essential to use this type of mixer which has a paddle rotating with a planetary motion, otherwise it was not possible to get a uniform pH throughout the comparatively stiff meat-water paste. After 15 min. mixing the pH of the meat was determined and the meat then stored at 2–4°. During this time the pH slowly fell, as is shown in Fig. 1. Curve *A* shows the pH of the mixtures immediately after the 15 min. mixing, curve *B* after 2 hr. and curve *C* after 14 hr. in the cold room. After 14 hr. there was very little further change in the pH. The values shown in curve *A* were not closely reproducible even if meat of the same initial pH was used, but it was found that the amount of toxin subsequently produced from digests of the meat was dependent not so much upon the pH to which the meat was adjusted as upon the amount of alkali added to the meat.

After the various samples of meat had stood for 48 hr. at 2–4° 1 g. of commercial papain (Thomas Morson) was mixed with each and the temperature was raised to 60° and kept there with occasional stirring for 3·5 hr. The meat rapidly liquefied. After the digestion the pH was lowered to 5·5 and the digests were boiled for 10 min. and filtered. These filtrates (*c.* 1250 ml./2 kg. meat) generally contained about 15 g. total N/l., 3 g. amino N/l., 2 g. non-dialyzable total N/l., and 10–13 mg. available Fe/l. The available iron was decreased to 200–300 μg./l. by adjusting the pH to 7·8, adding 3 mmol. $CaCl_2$/l., boiling and filtering. The filtrates were then diluted to contain 6 g. total N/l., 1% $FeSO_4 . 7H_2O$ was added to bring the available iron concentration up to 1200 μg./l. and the following reagents added/l.: 7·5 g. sodium glycerophosphate; 6 g. glucose; 0·1 ml. thiolacetic acid. The pH was adjusted to 7·2 and the medium sterilized by filtration. Each batch of medium was inoculated with a fresh culture of *Cl. welchii*, type A, strain S 107, and incubated at 37° for 5 hr. The cultures were then filtered and the α-toxin content of the filtrates determined. The results are plotted in Fig. 2. The data in this curve are reproducible in that optimum toxin production is always obtained when 80 ml. N-NaOH are used for every 2 kg. meat, but the value 4·72 Le/ml. is higher than average; generally 3–3·5 Le/ml. are obtained. If the pH of the meat rather than the amount of alkali added were plotted on the abscissa the curve would not be reproducible. An attempt was made to prevent the pH of the meat from changing while standing at 2–4°, by mixing in $CaCO_3$, but no improvement in toxin production was observed. It was also found that there was no

change in toxin production if the alkali-treated meat was allowed to stand for as long as 6 days. Toxin production was always slightly lower if the meat stood for only 1 day.

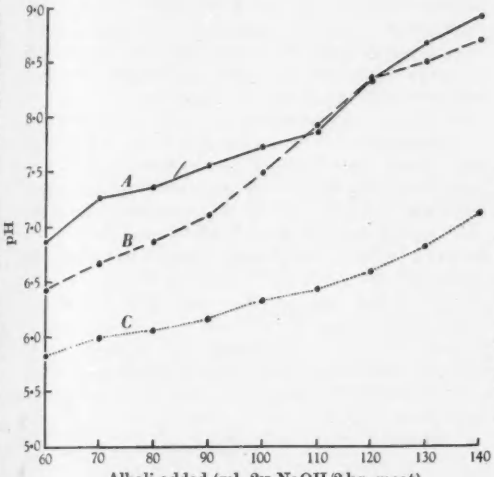

Fig. 1.

Fig. 1. Effect of alkali on the pH of meat. Time at 2–4° after addition of alkali: *A*, 15 min.; *B*, 2 hr.; *C*, 14 hr.

It is important that the digestion of the meat should be carried out with the concentration of meat as high as 2 kg./l. of added water. When larger quantities of water were used the toxin production was invariably lower; with a ratio of meat to water of 1:1 the toxin production was less than half.

It is an advantage to add the papain to the meat while the alkali is being mixed in; this does not affect toxin production, but it simplifies the digestion procedure. If papain is initially present the meat starts liquefying while the temperature is being raised to 60° for the digestion, and is consequently more easily manipulated and stirred than it would be without papain. Without the papain there is a tendency for the meat to overheat. When crude pepsin and pancreatic extract were used instead of papain a medium was obtained upon which about half as much toxin could be obtained as on a papain digest.

The addition of protein-free horse-muscle extract (Macfarlane & Knight, 1941) to media prepared in this way did not increase toxin production, in fact it always had a slight inhibiting effect.

Pappenheimer (private communication; cf. Pappenheimer & Shaskan, 1944) had found that with a medium he was using the concentration of available iron had a marked effect on α-toxin production. The concentration of available iron was also found to influence the α-toxin production in the deferrated

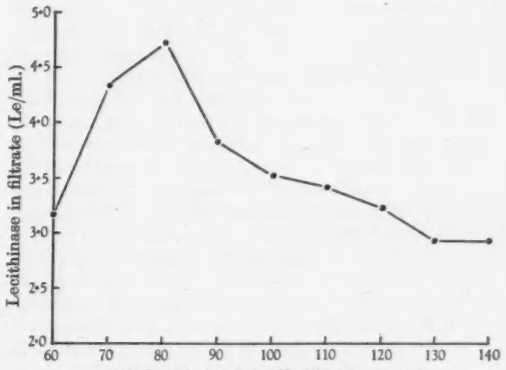

Fig. 2.

Fig. 2. Effect of alkali during pre-treatment of meat, on yield of lecithinase.

medium described above, but not to a great degree, as will be seen in Fig. 3. There appears to be an optimal iron concentration at 1000–1200 μg./l. Later it was found that if the medium was not deferrated it

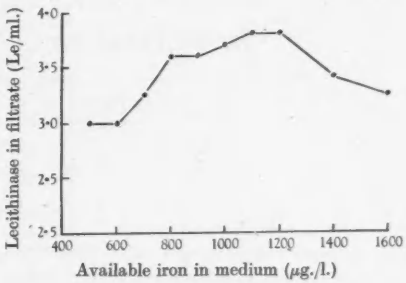

Fig. 3. Effect of iron, added to deferrated media, on yield of lecithinase.

was not affected by the concentration of available iron, and as high concentrations of toxins could be produced on such media, containing about 4000 μg. available Fe/l., as could be obtained on deferrated

media to which iron was added to the optimal concentration of 1200 μg./l. It is possible that during deferration some substance or substances which renders the organism less susceptible to the available iron is removed in addition to the iron.

DISCUSSION

On a medium produced in the laboratory by the methods described above culture filtrates containing 3·5–5 Le units of lecithinase/ml. can be obtained with satisfactory regularity. The yield of θ-haemolysin varies from 2 to 15 units/ml., and that of collagenase between 4 and 6 κ units/ml. The medium has successfully been produced on the large scale by Mr J. G. C. Campbell of these laboratories with such modifications as production procedures entail. During 12 months more than 6000 l. of this medium were produced, generally in batches of 200–300 l., for the production of lecithinase. The yield of lecithinase was generally between 2·5 and 4 Le/ml.

It is not yet known why the meat should have to receive the alkali treatment before digestion, or why the digestion should have to be carried out with a high concentration of meat. It is possible that during the period of standing, during which the pH passes through neutrality, autolysis takes place under the conditions observed and toxigenic substances are set free. Rogers & Knight (1946) have shown that autolysis of meat favours the production of substances which stimulate lecithinase production by *Cl. welchii*. The high concentration of meat during digestion might be necessary in order that an adequate concentration of toxigenic substances may be extracted from the meat in relation to the concentration of other nutrient and toxigenic substances liberated by the digestion.

An empirical medium such as the one described in this paper is obviously unsatisfactory in the long run. It savours more of the kitchen than of the laboratory, since there are a number of uncontrollable and unknown factors in its production and in its constitution. The development of a medium of which the constituents are known is obviously desirable.

SUMMARY

The development of an empirical medium suitable for the large-scale production of the α-toxin (lecithinase) of *Clostridium welchii*, type A, is described.

I am grateful to Mr R. N. Beale and to Miss E. Bidwell for much assistance and to Dr B. C. J. G. Knight and Mr J. G. C. Campbell for helpful discussion and advice.

REFERENCES

Macfarlane, M. & Knight, B. C. J. G. (1941). *Biochem. J.* **35**, 884.
Macfarlane, R. G., Oakley, C. L. & Anderson, C. G. (1941) *J. Path. Bact.* **52**, 99.
Oakley, C. L., Warrack, G. H. & van Heyningen, W. E. (1946). *J. Path. Bact.* **58**, 229.
Pappenheimer, Jr., A. M. & Shaskan, E. (1944). *J. biol. Chem.* **155**, 265.
Pope, C. G. & Stevens, M. F. (1939). *Biochem. J.* **33**, 1070
Rogers, H. J. & Knight, B. C. J. G. (1946). *Biochem. J.* **40**, 400.
van Heyningen, W. E. (1941*a*). *Biochem. J.* **35**, 1246.
van Heyningen, W. E. (1941*b*). *Biochem. J.* **35**, 1257.

The Biochemistry of the Gas Gangrene Toxins

4. THE REACTION BETWEEN THE α-TOXIN (LECITHINASE) OF *CLOSTRIDIUM WELCHII* AND ITS ANTITOXIN

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If constant amounts (300 units) of a sufficiently avid diphtheria antitoxin (horse serum) are added to a series of tubes containing increasing amounts of diphtheria toxin, ranging from 150 to 450 Lf units, flocculation will take place in each tube and the supernatants will be very nearly immunologically neutral. The ratio of toxin to antitoxin in the flocculs will vary from TA_2 to T_3A_2, where T is an Lf unit of toxin and A is a unit of antitoxin. If, (*a*) no other antigen-antibody systems are involved in the flocculation, (*b*) no impurities associated with the toxin or antitoxin preparations are adsorbed on the floccules, and (*c*) the toxin-antitoxin floccules are quantitatively precipitated, then the floccules should contain all the toxin and all the antitoxin added to each tube, and nothing else. The difference

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between the nitrogen contents of the floccules from the mixtures containing 150 and 450 toxin units will represent the N content of 300 toxin units. In other words, if the N content of the floccules is plotted on the ordinate against the units of toxin employed on the abscissa, a straight line will be obtained whose slope will represent the N content of one unit of toxin and whose intercept with the ordinate will represent the N content of 300 units of antitoxin.

By employing a technique involving the principles outlined above Pappenheimer & Robinson (1937) concluded that the N content of 1 Lf unit of diphtheria toxin is 0·00046 mg., and of 1 unit of antitoxin is 0·0016 mg. This figure of 0·00046 mg. N/Lf agrees very well with the figure of 0·00045 mg. N/Lf which Pappenheimer (1937) later found by direct estimation for pure diphtheria toxin. Since the figure given by Pappenheimer & Robinson was obtained irrespective of the purity of the toxin and antitoxin preparations* they used, we had hoped, in the course of our work on the purification of the α-toxin of *Clostridium welchii*, to employ a similar technique for determining the percentage specifically precipitable N, i.e. the percentage purity, of our toxin preparations. Our experience shows, however, in the case of *Cl. welchii* α-toxin at any rate, that non-specific impurities are adsorbed on the toxin-antitoxin floccules. We have also observed that different batches of toxin behave differently in the toxin-antitoxin reaction.

METHODS

Estimation of toxin and antitoxin. Toxins and antitoxins were estimated either by measuring their antitoxin and toxin combining power (Le/ml. for toxins) with crude lecithin as indicator for slight toxin excess (see van Heyningen, 1948), or by measuring their flocculating power (Lf/ml. for toxins). For most of the work the latter method was used. The primary standard was the British Standard Perfringens Antiserum (National Institute for Medical Research). The Lf units/ml. of a toxin preparation, Pf 13D, were determined against this standard, and a substandard antitoxin preparation, RX 5167 (which was pepsin-refined and which was also the source of antitoxin used in all the experiments described in this paper) was assayed against it. All other toxin preparations were assayed against the substandard antitoxin preparation. In all assays increasing amounts of antitoxin were mixed with constant amounts of toxin ('β-procedure') and the order of flocculation observed. If the flocculations are carried out at 40° rather than 50° a better accuracy is obtained, since the time intervals at which the different mixtures flocculate are longer. Conditions were so arranged that the indicating tube contained about 40 units each of the reactants in a volume of about 2·5 ml., and that the mixture flocculated in about 7 min.

For each assay at least 25 tubes in duplicate were used in which the amounts of antitoxin differed by 2% increments. The tubes were compared five (sometimes seven) at a time according to the scheme below:

A	B	C	D	E
1	6	11	16	21
2	7	12	17	22
3	8	13	18	23
4	9	14	19	24
5	10	15	20	25

The serial numbers represent tubes in which the amounts of antitoxin increase by 2%; the horizontal rows of numbers represent the sets of tubes observed at one time.

Each set of five tubes was set up in duplicate. Thus five duplicate observations were made in which the spacing between the successive amounts of antitoxin was 10%, the final effect being that the relative order of flocculation of mixtures containing antitoxin with 2% spacing was obtained. According to the order of flocculation of three adjacent mixtures, B, C and D, the amount of antitoxin which was considered equivalent to the constant amount of toxin used was judged as follows:

Order of flocculation	Equivalent amount of antitoxin
C, then B and D simultaneously	C
B, C and D simultaneously	C
C and D simultaneously	Mean of C and D
B and C simultaneously	Mean of B and C
C, then D	C + 25% of difference between C and D
C, then B	C − 25% of difference between C and B

By this means values with an error of ± 2% or less could be obtained 19 times out of 20. Thus the value of the toxin preparation Pf 13D was found to be 1234 ± 11 Lf/ml., and that of the antitoxin preparation RX 5167 was 4462 ± 73 units/ml.

Diffusion coefficients. The diffusion coefficient of α-toxin and of the pigment associated with it in some preparations was measured by the method of Northrop & Anson (1929). The enzyme and the pigment were allowed to diffuse at 3·5° into borate buffer pH 6·7, and the Le or pigment content was measured at daily intervals. Two diffusion cells were used, one of which was kindly lent to us by Dr Roy Markham. The constants for the cells were determined with carbon monoxide haemoglobin, and

* Pappenheimer & Robinson (1937) used both crude and pepsin-refined antitoxic globulin; the N content/unit of the latter was lower than the former. See also Pope & Healey (1939).

were 0·0641 and 0·378. On the advice of Dr Markham the diffusion coefficient, D_{20}, of CO haemoglobin was taken as 0·0553 cm.²/day.

Other estimations. Nitrogen was determined by micro-Kjeldahl procedure and pH was determined electrometrically with a glass electrode.

EXPERIMENTAL

Partial purification of α-toxin

The toxin preparations used were derived from filtrates of cultures of *Cl. welchii* type A, strain S 107, grown on the medium described by van Heyningen (1948).

Purification by ammonium sulphate fractionation. Concentrated and partially purified (at most 10% pure) preparations were routinely obtained from the crude filtrates according to the steps outlined in Table 1. The figures in Table 1 are the means from 22 experiments, and in each case they have been brought to the nearest round number.

Purification by nucleic acid precipitation. The Warburg school have recently used nucleic acid with great success in the isolation of enzymes from yeast (Warburg & Christian, 1939) and from tumour tissue (Kubowitz & Ott, 1943). We therefore applied this reagent to the purification of α-toxin. The starting material was preparation 3 of Table 1, i.e. the dialyzed solution of the $(NH_4)_2SO_4$ precipitate of the toxin from the crude filtrate. It was found to be essential that the preparation should be dialyzed. It was then treated in the following way:

(1) Crude toxin solution (4 l.) was cooled to 2–3° and held there. A neutral solution containing 4 g. yeast nucleic acid was added, and the solution carefully adjusted to pH 4·5 by adding about 320 ml. N-acetic acid over a period of 1 hr. with stirring. The acidified solution was left to stand at 2–3° for 2 hr. during which time a small dark brown precipitate settled out and was collected. The supernatant solution contained most of the κ-toxin (collagenase, see Bidwell & van Heyningen, 1948) and was therefore

Table 1. *Partial purification of α-toxin by ammonium sulphate fractionation*

Preparation	Volume (l.)	Treatment	Le*/ml.	Overall yield (%)	Le*/mg. non-dialysable N	Overall purification
1 (crude filtrate)	200	Stir in 120 kg. $(NH_4)_2SO_4$. After standing overnight pick off the firm scum from the surface, dissolve in water and filter. pH 6·5	3	—	5	—
2	2	Dialyze 3 days against running tap water	240	80	15	3-fold
3	4	Stir in 1·68 kg. $(NH_4)_2SO_4$. After 1 hr. collect precipitate and dissolve in water. pH 6·5	114	76	60	12-fold
4	0·50	Dialyze overnight against running tap water	820	69	120	24-fold
5	0·75	Add 0·8 vol. saturated $(NH_4)_2SO_4$ solution. After 1 hr. collect precipitate and dissolve in water. pH 6·5	520	65	120	24-fold
6	0·25	Store or use	1150	49	250	50-fold

* Le = lecithinase antigen units.

The final product, of which preparation Pf 13 D is an example, was highly pigmented—so much so that it was impossible to examine it in the electrophoresis apparatus. The yield and degree of purification obtained by this procedure were much the same as those obtained by an earlier procedure which involved adsorbing the toxin on calcium phosphate in the presence of acetone (van Heyningen, 1941). The present procedure can more conveniently be applied to large volumes of culture filtrate. Further treatment of the final product with $(NH_4)_2SO_4$, organic solvents, or adsorbents failed to yield a purer preparation. Preparations such as these were used for the production of concentrated toxoids in an investigation of the possibilities of active immunization against gas gangrene. For convenience these preparations will be called AS preparations.

readjusted to pH 7 and stored. The precipitate was suspended in 40 ml. water and N-NaOH carefully added with stirring until the pH was 6·0; the precipitate then dissolved completely to give a very dark brown solution containing the α-toxin in 50% stage yield. The solution contained nucleic acid, some of which presumably was bound to the toxin.

(2) Nucleic acid, and probably other impurities, can be removed by the addition of protamine until no further precipitate is obtained. It is not possible to calculate how much protamine to use, and therefore it was necessary to carry out a preliminary titration on a sample of the toxin-nucleic acid solution. The amount of protamine sulphate required to work up 200 l. of crude filtrate varied from 1·5 to 4 g. It was added in the form of a 2% solution of salmine sulphate (Wellcome Chemical Works) at pH 6. At this pH the solution was very opalescent and probably some of the protamine was carried in suspension. A considerable amount of pigment was removed by the protamine pre-

cipitation, leaving a light yellow supernatant solution containing the α-toxin in 60–80% stage yield.

(3) After the protamine nucleate precipitate had been removed by centrifugation excess protamine and some other impurities, including pigment, were removed by treating the solution with 1/8th volume of 30% alumina cream suspension (i.e. 30% solid by volume). The stage yield of α-toxin was 75%, and the overall yield from crude filtrate to this stage about 20%. The final product was nearly white when dry and light yellow in 2% solution. Upon electrophoresis (kindly done for us by Mr P. A. Charlwood) the preparation appeared to contain four components, the most abundant of which amounted to about 50% of the total solids. These preparations contained 1200–1500 Lf/mg. N, and about 10,000 average lethal doses (mouse, intravenous)/mg. As will be seen later, these preparations cannot be more than 50% pure; the overall purification with respect to crude filtrate was 250-fold, or 5-fold with respect to the AS preparations previously mentioned. Compared with crude filtrate the ratio of κ-toxin to α-toxin in the nucleic acid purified preparations was decreased about 30-fold; the hyaluronidase/α-toxin ratio was decreased about 2000-fold; the θ-toxin/α-toxin ratio remained almost unchanged. For convenience these preparations will be referred to as NA preparations. The diffusion coefficient of the α-toxin, D_{20}, was found to be 0·064 cm.²/day. We were not tempted to calculate the molecular weight of the toxin from its diffusion coefficient.

The toxin-antitoxin reaction

The diphtheria toxin-antitoxin reaction has been studied by three techniques:

(i) Pappenheimer, Lundgren & Williams (1940) made an ultracentrifugal study of the toxin-antitoxin complexes in solution in excess toxin and antitoxin. From the sedimentation characteristics of the components formed in these regions they deduced the existence of complexes ranging from TA_4 to T_4A. (T and A represent units of toxin and antitoxin respectively. The expression TA_4, to take an example, does not necessarily represent a single complex, but the unit ratio of toxin to antitoxin in a mixture of, say, two parts of TA_5 and one part of TA_2. It is in this sense that the expressions T_xA_y are used throughout this paper.)

(ii) From determinations of the residual toxin or antitoxin contents of mixtures of toxin and antitoxin, in which precipitation may or may not have occurred, Healey & Pinfield (1935) concluded that complexes ranging from TA_2 to T_3A_2 were formed. They were not able to detect complexes containing more antitoxin than in TA_2, or more toxin than in T_3A_2. Evidently antitoxin in excess of the ratio TA_2 reacts rapidly with added toxin to form TA_2, whereas TA_2 reacts slowly with T to form TA.

Thus
$$TA_4 + T \xrightarrow{\text{fast}} 2TA_2,$$
$$TA_2 + T \xrightarrow{\text{slow}} 2TA$$
and similarly
$$3T_4A + 5A \xrightarrow{\text{fast}} 4T_3A_2,$$
$$T_3A_2 + A \xrightarrow{\text{slow}} 3TA.$$

(iii) By determining the nitrogen in the floccules precipitated from toxin-antitoxin mixtures Pappenheimer & Robinson (1937) made a quantitative study of the formation of insoluble toxin-antitoxin complexes.

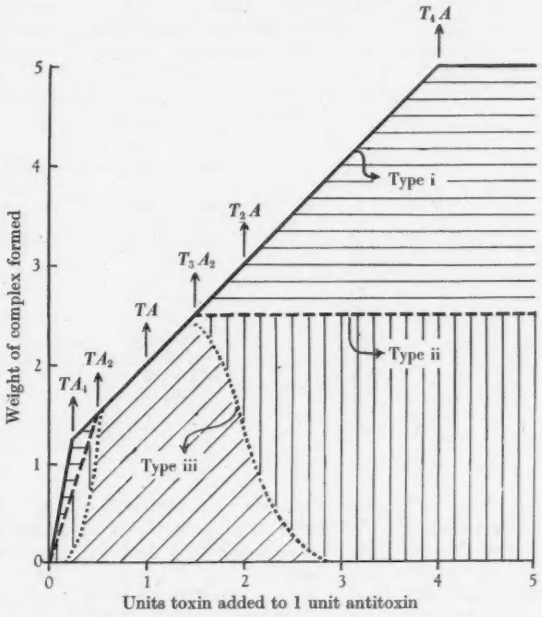

Fig. 1. Weights of complexes formed by interaction of toxin and antitoxin of arbitrary unitage and weight. Type i, total complexes; type ii, soluble and insoluble slow-reacting complexes; type iii, insoluble complexes. (See text for explanation.)

The information gained from the application of these three techniques is illustrated in Fig. 1, in which are plotted the weights of toxin-antitoxin complexes formed, using arbitrary units and taking a weight of 1 for each unit. The type of graph obtained by each technique is correspondingly marked; the horizontally hatched areas show the soluble toxin-antitoxin complexes which react rapidly with toxin or antitoxin to form the complexes shown in the vertically hatched areas; the vertically hatched areas show the soluble complexes which react slowly; the diagonally hatched area shows the insoluble complexes, which also react slowly.

We have made a similar study of the *Cl. welchii* α-toxin-antitoxin reaction with the latter two techniques ((ii) and (iii) above).

Titration of supernatants

Increasing amounts of the toxin preparation Pf 13D were mixed with constant amounts (242 units) of antitoxin, in a constant volume of 6 ml. After 3 days standing at 4° the floccules which precipitated in all the mixtures were spun down and the supernatants titrated for toxin and antitoxin with egg yolk as indicator. The results obtained are shown in Table 2. From these figures it appears that with the preparation Pf 13D complexes ranging from TA_2 to T_2A are detectable. The preparation Pf 13D is exceptional, however; with most batches of toxin the range is approximately T_2A_3 to T_6A_5, i.e. the ratio of toxin to antitoxin in the complexes covers about a twofold range. Mixtures of toxin and antitoxin were made containing either 12,000 units of antitoxin and 3000 units of toxin, or 12,000 units of toxin and 4000 units of antitoxin, in 180 ml. volumes, involving altogether 10 different batches of toxin and one batch of antitoxin. In none of these mixtures was there any precipitate formed. After standing overnight at 4° the free antitoxin or toxin was determined. The titrations showed that on the antitoxic side the complexes detected varied from $TA_{1\cdot45}$ to $TA_{1\cdot64}$, and on the toxic side from TA to T_3A_2, most of the toxic complexes having the composition T_6A_5.

From the work of Healey & Pinfield (1935) it is evident that complexes of the type TA_2 react slowly with toxin to form TA. We have found that α-toxin-antitoxin complexes will react in a similar manner, but that again there is variation with different batches of toxin. Mixtures were made containing 12,000 antitoxin units and 3000 toxin units in a volume of 180 ml. After standing overnight at 4° the free antitoxin was determined as follows: increasing amounts (spaced 10%) of the antitoxic mixtures were added to constant amounts (1 Le unit) of test toxin. Several similar series of such mixtures were made. Pairs of these series were left to stand for 30 min. (as in the usual assay), 1 day and 2 days before the egg-yolk solution was added and the mixtures incubated at 37° for 1 hr. The longer the antitoxic mixtures were allowed to stand with the test toxin the more toxin they combined with, i.e. the greater was their apparent antitoxin content. The results obtained with three different batches of toxin are shown in Table 3. Batches B 195G and B 197G behaved much as the samples of diphtheria toxin studied by Healey & Pinfield (1935). The interesting behaviour of batch B 202J

Table 2. *Titrations of free antitoxin or antitoxin in mixtures containing increasing amounts of toxin and 242 units of antitoxin*

I Toxin added (units)	II Free antitoxin (A) or toxin (T) in supernatants (units)	III Units antitoxin (A) combined with units toxin in col. I or units toxin (T) combined with 242 units antitoxin	IV Ratio T/A in complex	
95	72 (A)	170 (A)	0·55	
119	6·5 (A)	236 (A)	0·50	
166	1·2 (A)	241 (A)	0·69	
214	0·3 (A)	242 (A)	0·88	
242	<0·01 (T)	242 (T)	1·00	
363	<0·01 (T)	363 (T)	1·50	
484	<0·01 (T)	484 (T)	2·00	
605	144 (T)	461 (T)	1·90	Mean 1·98
726	264 (T)	462 (T)	1·91	
847	396 (T)	451 (T)	1·86	
965	432 (T)	533 (T)	2·20	

Table 3. *Slow reaction between T and complexes containing more A than in ratio $T/A = 1$*

Toxin	Units toxin added to 12,000 units antitoxin	Apparent free antitoxin units on 30 min. test	Apparent units antitoxin combined	Ratio T/A in complex detected on rapid T-A reaction	Apparent free antitoxin units on 2-day test	Apparent units antitoxin combined	Ratio T/A in complex detected on slow TA_x-T reaction
B 195G	3,000	7,650	4,350	0·69	9,370	3,630	0·83
B 197G	3,170	6,800	5,200	0·61	8,500	3,500	0·91
B 202J	3,000	7,650	4,350	0·69	10,500*	1,500	2·00

* 1-day test.

will be referred to again in the discussion. It would have been interesting to study the reaction of complexes containing higher ratios of toxin than *TA*, and batch Pf 13D with which a ratio of T_2A was detected would have been very suitable; unfortunately, it had all been used by the time we realized that these experiments should be done. With the other batches of toxin complexes of average composition T_6A_5 were detected and the difference between T_6A_5 and *TA* is too small to allow significant changes to be observed.

Quantitative flocculation

The optimum conditions for quantitative flocculation were determined.

Effect of temperature. Mixtures containing equivalent amounts of toxin and antitoxin made up to 6 ml. with 1 % NaCl were (*a*) left at 4° for 4 days, (*b*) left at 4° for 4 days, the floccules then resuspended, kept at 50° for 3 hr., and then left at 4° for another day, (*c*) kept at 50° for 3 hr., then left at 4° for 4 days. The floccules were then spun down in a refrigerated centrifuge, washed twice with 6 ml. chilled saline and their N content measured. The results of duplicate determinations are shown in Table 4. Since no significant differences resulted from the different temperatures at which the mixtures were kept, mixtures hereafter were always left in the cold room, without treatment at 50°.

Table 4. *Effect of temperature on flocculation*

Units toxin and antitoxin	Nitrogen in floccules (mg.)		
	4 days at 4°	4 days at 4°, 3 hr. at 50°, 1 day at 4°	3 hr. at 50°, 4 days at 4°
100	0·296	—	0·281
	0·278	0·307	0·261
200	0·616	0·612	0·597
	0·625	0·597	0·598
300	—	—	0·932
	0·934	0·907	0·945
400	1·236	1·232	1·236
	1·281	—	1·227
500	1·600	1·549	1·586
	1·591	—	1·528

Effect of concentration of reactants on flocculation. Mixtures containing 190 Lf units of toxin and 242 units of antitoxin were made up to different volumes and left at 4° for 5 days. The floccules were then washed twice with 6 ml. chilled saline and their N content determined. The results are shown in Table 5. It will be seen that within a range of nearly threefold the concentration of toxin-antitoxin does not significantly affect the amount of precipitate formed.

Table 5. *Effect of concentration on flocculation*

Volume (ml.)	3	4	5	6	7	8
Nitrogen in floccules (mg.)	0·783	0·757	0·751	0·756	0·825	0·744
	—	0·761	0·754	0·767	—	0·748

Effect of time of standing and of washing on floccules. Identical mixtures containing 190 Lf units of toxin and 180 units of antitoxin made up to 6 ml. in saline were left at 4° for different times, and then the floccules were spun down and washed 2 or 3 times with 6 ml. volumes of chilled saline. The results are shown in Table 6. They suggest that possibly there was a very slight increase in the amount of N precipitated in 3 days as compared with 1 day. They do not show any significant difference between two and three washings (see also Table 9).

The quantitative study of the flocculation reaction was carried out as follows: Appropriate amounts of toxin and antitoxin were carefully pipetted into 15 ml. centrifuge tubes, thoroughly mixed, and the volumes made up to 6 ml. with 1 % NaCl solution. The tubes were then fitted with rubber caps and left at 4° for 4 days. Then the floccules were spun down in a refrigerated centrifuge, resuspended in 6 ml. chilled saline with the help of a small high-speed stirrer, spun down and washed a second time. After being spun down for the last time the floccules were Kjeldahl-digested in their centrifuge tubes.

Table 6. *Effect of time of standing, and of washing on amount of floccules obtained*

Time (days) ...		1	2	3	5	6
Nitrogen in floccules (mg.)	2 washings	0·596	—	0·615	0·626	0·560
		0·601	—	0·612	0·637	0·633
	3 washings	0·595	0·596	0·600	0·627	0·602
		—	0·593	0·589	0·627	0·613

The experiments done with toxin preparation Pf 13D are typical. Increasing amounts of toxin, varying from 95 to 965 Lf units, were mixed with constant amounts (242 units) of antitoxin and the N content of the floccules determined. The results are plotted in Fig. 2. (The point corresponding to

95 toxin units is the mean of four determinations, the points in the equivalence zone from 118·5 to 237 toxin units are each the mean of ten determinations; the points in the range 363–965 toxin units are each the mean of four determinations.) The mean values for the equivalence zone are given in Table 7. In the equivalence zone the supernatants contain neither free toxin nor free antitoxin. The points in the equivalence zone lie on a straight line, the slope of which is the amount of nitrogen associated with one Lf unit of toxin, and the intercept of which with the ordinate is the amount of nitrogen associated with the total number (242) of antitoxin units used. These relationships are given by the expression $y = a + bx$, where y = N in flocculs, a = N associated with antitoxin in flocculs, b = N associated with 1 toxin unit in the flocculs, and x = number of toxin units.

In all the experiments of this type we have calculated the slope (b) of the best straight line passing through the points in the equivalence zone, and from that its intercept (a) with the ordinate, by the usual statistical methods. For convenience the figure for the amount of N associated with one Lf unit of toxin in the floccules will be called the 'slope' of a given toxin preparation, and the corresponding figure for the amount of N associated with one unit of antitoxin will be called the 'intercept'. Thus for the toxin preparation Pf 13D the 'slope' was found to be 0·00201 mg. N/Lf toxin, and the 'intercept' was found to be 0·00146 mg. N/unit antitoxin.

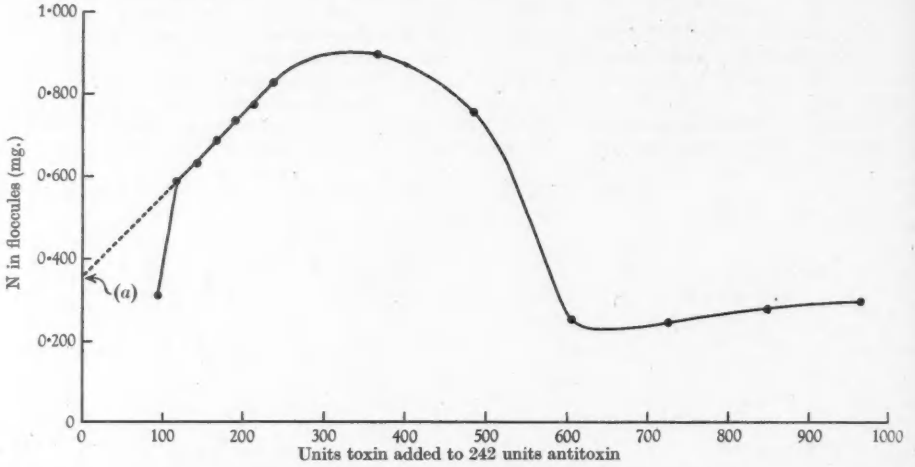

Fig. 2. Nitrogen in floccules precipitating from toxin-antitoxin mixtures.

Fig. 3 shows that essentially the same curves were obtained when the nitrogen in the floccules was either determined directly, or calculated from the difference in the N content of the toxin-antitoxin mixtures before and after flocculation. (Each point is the mean of three or four determinations.) The values found by difference were consistently slightly higher than those found directly.

The curves in Figs. 2 and 3 are much the same as that found for diphtheria toxin by Pappenheimer & Robinson (1937). The main difference between our curves and theirs is that we still found appreciable

Table 7. *Mean values for equivalence zone in Fig.* 2

I	II	III	IV	V
x Units toxin	Ratio A units/T units	bx Toxin N (mg.)	y Ppt. N (mg.)	a Antitoxin N in ppt. (mg.) (IV – III)
118·5	2·04	0·239	0·593	0·354
142·5	1·70	0·286	0·638	0·352
166	1·46	0·334	0·689	0·355
190	1·27	0·382	0·737	0·355
213·5	1·14	0·430	0·784	0·354
237	1·02	0·478	0·830	0·352

precipitates when the ratio of toxin to antitoxin units was as high as 4 : 1, whereas in the diphtheria system there was no precipitate beyond the ratio 2 : 1. Pope & Healey (1938) point out that diphtheria antitoxin modified by treatment with pepsin (Pope, 1938) flocculates over a wider range than does untreated antitoxin.

batches of AS toxin preparations we found that, although most of them gave approximately the same 'slopes', the 'intercepts' varied from batch to batch. Table 8 shows the values obtained for five batches of AS toxin preparations. Batch B 197G is exceptional. That these differences in 'intercepts' are not due to batch to batch variations in the

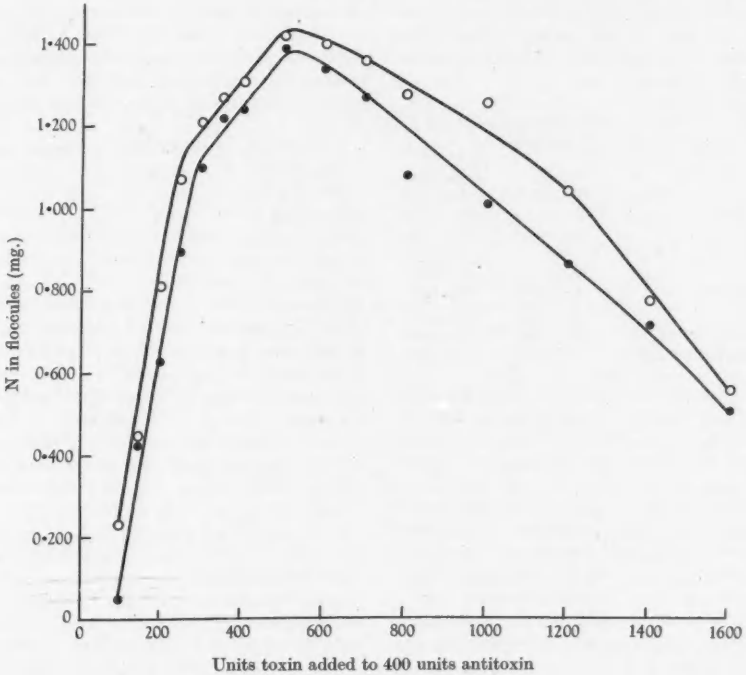

Fig. 3. Nitrogen in floccules precipitating from toxin-antitoxin mixtures. Upper curve, as deduced from difference in N contents of mixtures before and after flocculation; lower curve, as determined directly on the floccules.

Table 8. *Variations in 'intercepts' with different batches of AS toxin preparations*

Toxin preparation	'Slope' N associated with 1 Lf unit toxin (mg.)	'Intercept' N associated with 1 unit antitoxin (mg.)
B 195G	0·00185	0·00194
B 165G	0·00184	0·00184
Pf 13D	0·00201	0·00146
B 170G	0·00180	0·00080
B 197G	0·00079	0·00119

The 'slope' and 'intercept' for Pf 13D were determined at least five times, and the values obtained were never significantly different from 0·0020 and 0·00146 mg. N respectively; but when similar determinations were made on a number of different

Table 9. *Showing insolubility of floccules in saline*

Toxin preparation	N in floccules (mg.) Washed with 12 ml. saline	Washed with 40 ml. saline
B 158G	1·451	1·299
B 165G	1·328	0·966
B 166G	1·323	1·286
B 170G	1·315	1·007
B 174F	1·299	1·052
B 146A	1·233	1·309
Pf 199	1·126	1·299
Pf 219	1·025	1·186
B 195G	0·991	1·273
B 197G	0·774	0·752

solubility of the floccules in the saline wash liquors is evident from the figures in Table 9. For each of ten different batches of AS toxin preparations eight

identical mixtures containing 400 Lf units of toxin and 400 units of antitoxin in volumes of 10 ml. were set up. After 4 days at 4° the floccules were spun down. In each case four of the precipitates were washed twice with 6 ml. chilled saline, and the other four were washed four times with 10 ml. chilled saline. The N contents of the washed floccules were then determined. We do not think the figures show important differences between the N contents of floccules washed with 12 ml. saline and of those washed with 40 ml. saline. (Each figure is the mean of four determinations.)

Table 10. *Different 'slopes' obtained with AS and NA toxin preparations*

Toxin preparation		Lf/mg. N	'Slope' N associated with 1 Lf unit toxin (mg.)	'Intercept' N associated with 1 unit antitoxin (mg.)
AS	211 L	14·4	0·0021	0·00189
	Pf 13 D	273	0·0020	0·00146
NA	B 259 F	815	0·00039	0·00140
	B 144 B	1250	0·00040	0·00154
	B 163 A	1500	0·00045	0·00145

While the 'slopes' for AS toxin preparations generally did not differ appreciably from 0·0020 mg. N associated/unit toxin, we found that the 'slopes' for the purer NA toxin preparations were much lower. This is shown in Table 10, where it will be seen that even though the AS toxin preparation 211 L (which was precipitated once only with $(NH_4)_2SO_4$) was nearly 20 times as crude as the AS preparation Pf 13 D they both had the same 'slope'. Thus it seems that the impurities which can be removed by ammonium sulphate fractionation, and which represent the difference between the preparations 211 L and Pf 13 D, were not adsorbed on the toxin-antitoxin floccules. On the other hand, some of the impurities which were removed during the nucleic acid precipitation of the toxin appeared to be adsorbed on the floccules, as is evident from the difference of the 'slopes' for NA and AS preparations.

That this difference was due to adsorption of non-specific impurities is also suggested by the following experiment: A batch of filtrate from a profuse culture of *Cl. welchii*, type A, strain S 107, which had produced practically no α-toxin, happened to be available. It was subjected to the $(NH_4)_2SO_4$ fractionation outlined in Table 1 and the resultant product (which contained less than 5 Lf/ml.) was mixed with the NA preparation B 259 F, which contained 385 Lf/ml., 815 Lf/mg. N, and which gave a 'slope' of 0·00039 mg. N. The contaminated preparation now contained 40 Lf/mg. N, and on flocculation it gave a 'slope' of 0·00089 mg. N.

It is unlikely that the difference in the 'slopes' given by AS and NA preparations was due to a flocculating antigen other than α-toxin present in the AS preparations and not in the NA preparations. If this were the case one would expect the 'intercepts' for the NA preparations to be considerably lower than those of the AS preparations, since the antibody to the secondary antigen would not be precipitated in the flocculations with the NA preparations. In fact the 'intercepts' for AS and NA preparations are much the same. It is also worth recording that the AS preparation free from α-toxin used in the experiment just described did not precipitate with the antitoxin preparation used in this work.

At one time we entertained the suggestion that the change in 'slope' might be due to a part of the toxin molecule, amounting to one fifth of the whole and containing all the catalytic and antibody-combining centres, being split off during the nucleic acid purification. In such an event an approximately 1·7-fold change in the diffusion coefficient could be expected. We measured the diffusion coefficients of the toxin diffusing from AS and NA preparations and found a difference which we do not consider significant. The values of D_{20} for AS and NA preparations respectively were 0·059 and 0·067 cm.²/day. While we were measuring the diffusion coefficient of the toxin in the AS preparations we found that the pigment in these preparations also diffused at a uniform rate to give a value of $D_{20} = 0{\cdot}090$ cm.²/day.

DISCUSSION

The Danysz phenomenon results from the fact that 1 unit of toxin can combine with more than 1 unit of antitoxin, and from the fact that the complex TA_x (where $x > 1$) thus formed reacts slowly (whether or not it is in solution) with toxin to form TA. These two facts explain the phenomenon completely, and we do not see why it is more difficult to understand when the toxin-antitoxin complexes do not precipitate, and why Pappenheimer & Robinson (1937) should have found it necessary to introduce further assumptions in order to explain it. The fact that diphtheria toxin and α-toxin can also form slow reacting complexes of the type T_yA (Pf 13 D, Table 1, and B 202 J, Table 3, appear to form T_2A) suggests that what might be called a 'converse' Danysz phenomenon can also be expected.

We have seen that by titration complexes of the type TA_x to T_yA (where x and y both > 1) have been detected, and that the limits of x and y vary with different batches of toxin. It appears that the toxin and antitoxin in complexes of this type (i.e. those in the vertically hatched areas in Fig. 1) are involved in such a way that the reactant in excess of the ratio TA is not completely free to react with its immunological opposite and consequently it reacts slowly.

In complexes in which the values of x and y are greater than those observed in the titrations (i.e. complexes in the horizontally hatched areas in Fig. 1) the additional reactant is not so closely involved and reacts rapidly with its immunological opposite to form TA_x or T_yA as the case may be. Since the limits of x and y vary with different batches of toxin it seems that the way in which the toxin and antitoxin are bound together must vary in some way from batch to batch.

The toxin-antitoxin reaction takes place in two stages (see Boyd, 1943). The first stage consists of the union of toxin and antitoxin to form a soluble complex,

$$xT + yA \xrightarrow{\text{fast}} T_xA_y;$$

the second stage (which does not necessarily always follow) consists of the aggregation of the complex to form an insoluble precipitate,

$$T_xA_y \xrightarrow{\text{slow}} (T_xA_y).$$

We suggest that the reason why different batches of α-toxin appear to give different 'intercepts' might be that, although the first stage of the reaction is quantitative (since no free toxin or antitoxin is found in the supernatants), the second stage may not be quantitative and the degree to which it proceeds varies from batch to batch. Pope & Healey (1939) have found that diphtheria toxin-antitoxin floccules cannot always be centrifuged down quantitatively. With some samples of unrefined antitoxin as much as 50 % of the complex remained in the supernatant after prolonged centrifuging; the effect was much less marked with pepsin-refined antitoxin. They also found similar losses on washing the floccules with saline. Our floccules did not show a tendency to remain suspended after washing (Tables 6 and 9).

It seems reasonably clear, in the case of *Cl. welchii* α-toxin at any rate, that impurities from the toxin preparations can be adsorbed on the toxin-antitoxin floccules, and that such impurities can account for at least 30–45 % of the N in the floccules. Apparently no impurities were adsorbed on the diphtheria toxin-antitoxin floccules observed by Pappenheimer & Robinson (1937) since the 'slope' they found agrees exactly with the N content of a unit of pure toxin (Pappenheimer, 1937). Pope & Healey (1939), however, drew attention to the adsorption of pigments and lipids on diphtheria toxin-antitoxin floccules, and we found that the *Cl. welchii* α-toxin-antitoxin floccules tended to be pigmented, even after washing. Moloney & Hennessey (1942) found a 'probable value' of 0·0004 mg. for the 'slope' of tetanus toxoid; Pillemer, Wittler & Grossberg (1946) isolated pure tetanus toxin containing 0·00025–0·000286 mg. N/Lf. Hottle & Pappenheimer (1941), working with two partially purified toxin preparations, found a 'slope' of 0·00023 mg. N associated/Lf unit of scarlet fever toxin. There is no proof that impurities were not adsorbed on their floccules, and in view of our experience we think that such proof should always be provided. In the case of our toxin preparations it seems that there are at least two kinds of impurities. The first kind of impurity is removable by ammonium sulphate fractionation and is not adsorbed on toxin-antitoxin floccules (compare preparations 211L and Pf 13D, Table 10); the second kind of impurity is only removed when the toxin is precipitated with nucleic acid, and it is adsorbed on the floccules (compare AS and NA preparations, Table 10). Apparently this impurity was not adsorbed from preparation B 197G (Table 8) to the same extent as it was from other AS preparations. If the 'slopes' for the NA preparations represent the true value for the mg. N/Lf toxin, then the NA toxins are about 50 % pure, since the 'slopes' are about 0·0004 mg. N/Lf and the preparations contain about 0·0008 mg. N/Lf; but we have no proof that the floccules from these preparations also do not adsorb impurities and therefore we can only say that the preparations are not more than 50 % pure.

We have no means yet of knowing whether the adsorbable impurities are derived from the culture medium or from the organisms. The danger that such impurities may exist in any culture filtrate leads us to believe that only pure toxins and antitoxins can be used in quantitative flocculation studies. Although Pappenheimer & Robinson (1937) did not have trouble with adsorbable impurities in their diphtheria toxin preparations, such trouble might arise with other preparations of diphtheria toxin or of any other toxin. For this reason we do not think it safe to calculate the potency of impure toxin and antitoxin preparation from one (or more) nitrogen determination at the flocculation point as suggested by Pappenheimer & Robinson. We fear that in this respect we are on the side of the 'brass hats' (Heidelberger, 1946).

SUMMARY

1. Methods are given by which the α-toxin of *Cl. welchii* was routinely purified 50-fold by ammonium sulphate fractionation. By nucleic acid precipitation a further fivefold purification could be achieved. The final product was not more than 50 % pure.

2. The α-toxin was capable of combining with antitoxin to form, on the average, complexes containing toxin and antitoxin in ratios varying from T_2A_3 to T_6A_5 (where T and A represent Lf units of toxin and units of antitoxin respectively). These complexes, whether or not they were in solution, reacted slowly with toxin or antitoxin. With two batches of toxin, complexes of average composition TA_2 and T_2A have been detected.

3. Results obtained suggest that the formation of insoluble aggregates from soluble toxin-antitoxin complexes in the equivalence zone may not always be quantitative.

4. Impurities are adsorbed on the α-toxin-antitoxin floccules. The implications of this finding are discussed.

We are grateful to Miss A. G. Kennedy for many nitrogen determinations, to Miss Beryl Sievwright for much technical assistance, to Mr A. T. Glenny, F.R.S., and Mr H. Proom for the benefit of valuable criticism and stimulating discussion, and to Dr J. W. Trevan, F.R.S., for sympathetic encouragement. One of us (W.E.v.H.) also wishes to acknowledge the help given to him in Cambridge by Mrs E. E. Bacon.

REFERENCES

Bidwell, E. & van Heyningen, W. E. (1948). *Biochem. J.* **42**, 140.
Boyd, W. C. (1943). *Fundamentals of Immunology*, p. 164. New York: Interscience Publishers, Inc.
Healey, M. & Pinfield, S. (1935). *Brit. J. exp. Path.* **16**, 535.
Heidelberger, M. (1946). *Currents in Biochemical Research*, p. 453. New York: Interscience Publishers, Inc.
Hottle, G. A. & Pappenheimer, Jr., A. M. (1941). *J. exp. Med.* **74**, 545.
Kubowitz, F. & Ott, P. (1943). *Biochem. Z.* **314**, 94.
Moloney, P. J. & Hennessey, J. N. (1942). *Canad. publ. Hlth. J.* **33**, 157.
Northrop, J. H. & Anson, M. L. (1929). *J. gen. Physiol.* **12**, 543.
Pappenheimer, Jr., A. M. (1937). *J. biol. Chem.* **120**, 543.
Pappenheimer, Jr., A. M., Lundgren, H. P. & Williams, J. W. (1940). *J. exp. Med.* **71**, 247.
Pappenheimer, Jr., A. M. & Robinson, E. S. (1937). *J. Immunol.* **32**, 291.
Pillemer, L., Wittler, R. & Grossberg, D. B. (1946). *Science*, **103**, 615.
Pope, C. G. (1938). *Brit. J. exp. Path.* **19**, 245.
Pope, C. G. & Healey, M. (1938). *Brit. J. exp. Path.* **19**, 397.
Pope, C. G. & Healey, M. (1939). *Brit. J. exp. Path.* **20**, 213.
van Heyningen, W. E. (1941). *Biochem. J.* **35**, 1257.
van Heyningen, W. E. (1948). *Biochem. J.* **42**, 127.
Warburg, O. & Christian, W. (1939). *Biochem. Z.* **303**, 40.

The Biochemistry of the Gas Gangrene Toxins

5. THE κ-TOXIN (COLLAGENASE) OF *CLOSTRIDIUM WELCHII*

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Maschmann (1937) described an enzyme in young culture filtrates of *Clostridium welchii* (*B. perfringens*) which 'could attack gelatin extensively, but not ovalbumin, casein and fibrin'; he gave details for its partial purification (Maschmann, 1938*a*) and suggested the name 'collagenase' for it (Maschmann, 1938*b*), but later (Maschmann, 1938*c*) withdrew the suggestion without giving reasons.

The possible importance of a collagenase in gas gangrene was discussed by Maschmann (1937, 1938*a*, *b*) and the subject has been reopened recently by Macfarlane & MacLennan (1945). They observed in cases of clinical gas gangrene in man, and in rabbit muscle injected *in vivo* with *Cl. welchii* culture filtrates, that the trypsin-insoluble collagen framework of the muscle was disintegrated leaving the muscle fibres intact. Teased collagen from human tendon was lysed by *Cl. welchii* culture filtrate; these findings were confirmed by Robb-Smith (1945).

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Macfarlane & MacLennan suggested that *Cl. welchii* collagenase might be responsible for the liberation from the tissues of the host of a non-specific toxic substance(s) which might play an important part in gas gangrene toxaemia. Evans (1945) found that the anti-collagenase titre of *Cl. welchii* type A antisera was not correlated with the protection of guinea pigs against experimentally produced gas gangrene by the sera. At the suggestion of Drs Macfarlane and MacLennan we undertook the work to be described in this paper.

Oakley, Warrack & van Heyningen (1946) showed by titration with a large number of *Cl. welchii* antisera that the muscle-disintegrating substance present in *Cl. welchii* type A filtrates was distinct from α-toxin, θ-toxin and hyaluronidase, and that it was antigenically indistinguishable from the substance in those filtrates which attacks collagen in the form of collagen 'paper', hide powder and 'azocoll'. They proposed that the antigen concerned should be called κ-toxin.

We have succeeded in obtaining preparations of this collagenase free from other toxins of *Cl. welchii* and in a highly purified condition. In addition to its possible clinical interest, this collagenase is remarkable for its high substrate specificity, since it appears to attack only collagen and gelatin.

METHODS

Estimation of collagenase

The antibody-combining power, or antigen content, of collagenase preparations (expressed as κ units/ml.) was measured by the method of Oakley *et al.* (1946).

The enzymic activity (expressed as Q units/ml.) was determined by measuring the amount of dye liberated by the enzyme, under standard conditions, from hide powder to which R acid has been coupled ('azocoll', see Oakley *et al.* 1946). The Q unit is defined as that amount of enzyme which, under the conditions outlined below, will liberate twice as much dye from azocoll as is liberated in a blank.

Preliminary experiments with a photoelectric absorptiometer showed that the amount of dye liberated when a given amount of azocoll was shaken in 10 ml. buffer solution was sufficiently constant to be used as a colorimetric standard. The standard conditions chosen were as follows: 75 mg. of a given batch of 120-mesh azocoll were shaken for 1 hr. at 37° with 10 ml. buffer (equal vol. of M/15 phosphate buffer pH 7·4 and 1% NaCl). The reaction was stopped by filtering off undissolved azocoll. The amount of dye liberated, when enzyme solutions were incubated under the same conditions, was found to be a linear function of the enzyme concentration within the range 0·1–0·8 Q unit/ml., i.e. between the colorimetric ratios standard/unknown of 2–9, where the standard colour was that obtained in the blank as described above.

Since the amount of dye liberated varied with different batches of azocoll, it was important to use large batches. Most of our determinations were carried out with one batch of azocoll, and when a new batch was used it was necessary to determine its characteristics with a collagenase preparation the value of which had already been determined with the first batch. We cannot recommend the method since it cannot be truly standardized, and therefore we have not given it in detail. In our hands it was a rapid and fairly reliable means of following the progress of purification, which we regarded as an expedient to be discarded if a synthetic substrate were found.

Lecithinase (α-toxin) antigen was measured in Le units as described by van Heyningen (1948).

The efficiency of a procedure designed to separate collagenase from lecithinase was expressed by the ratio: Q units of collagenase/Le units of lecithinase in the product, to Q units/Le units in the original material.

Method of estimation of digestion of hide powder. Experiments were made with 120-mesh hide powder (specially prepared by Dr A. C. McDougall from hide powder supplied by Baird and Tatlock Ltd.) which had been washed with water and acetone and dried at 37°. Unless otherwise stated, the hide powder was incubated at 37° for 1 hr. in a total volume of 10 ml., in 25 ml. bottles which were agitated throughout that time. After incubation the mixtures were rapidly cooled to room temperature (within 0·5 min.) and filtered through Whatman no. 41 filter papers. Controls were run with corresponding heat-inactivated enzyme. Total N determinations were carried out on the filtrates. Results are usually shown as the difference between the total N of the filtrates when active and inactivated enzyme are used.

The action of the enzyme on gelatin. This was followed by determining the total N of the filtrate after the addition to the reaction mixture of 1/9th its volume of 5% (w/v) phosphotungstic acid (A.R.), in N-H_2SO_4.

Standard conditions obtained when 40 ml. of 0·5% (w/v) gelatin which had been incubated for 1 hr. at 37° with 5 ml. of enzyme solution were treated with 5 ml. precipitant. In experiments where gelatin solutions stronger than 0·5% were used, they were diluted at the end of the incubation, before adding the precipitant, so that the final concentrations were the same as for 0·5% gelatin. After thorough shaking, and standing for 5 min. the precipitates were filtered off on Whatman no. 3 papers. Heat-inactivated enzyme controls were treated in the same way. Results are shown as the difference between the total N of the filtrates when active and heat-inactivated enzyme preparations are used.

Other estimations. θ-Toxin by the method described by van Heyningen (1948); total nitrogen by micro-Kjeldahl procedure; NH_2-N by distillation in a Markham still (Markham, 1942) with 10 ml. of the phosphate buffer recommended by Sen (1942); 'tyrosine' by the method of Anson (1938); pH values were measured electrometrically with a glass electrode.

EXPERIMENTAL AND RESULTS

Purification of collagenase

The medium used in most of the work was a nutrient broth (4–5 g. total N/l.) containing 1% peptone, cooked meat and chalk. It was chosen, in preference to the medium described by van Heyningen (1948), because the resultant crude filtrate contains little if any θ-toxin. The organism was *Cl. welchii* type A, strain S 107.

Stage A. The culture filtrate was harvested after 5 hr. growth at 37°, the pH adjusted to 6·8, and $(NH_4)_2SO_4$ stirred in using 60 kg. $(NH_4)_2SO_4$/100 l. culture filtrate. After standing overnight the scum which floated on the surface was collected and dissolved in water. This solution will be referred to as the starting material.

The further stages in the purification were as follows:

Stage B. Adsorption on calcium phosphate followed by elution with 10% (w/v) $(NH_4)_2SO_4$.

Stage C. Charcoal treatment.

Stage D. Fractional precipitation with $(NH_4)_2SO_4$.

An outline of the procedure finally adopted may now be given.

Starting material from stage A was diluted to a collagenase concentration of 12–15 *Q* units/ml. and $(NH_4)_2SO_4$ added to a final concentration of 3·75% (w/v). A calcium phosphate precipitate was formed in the solution by adding 80 ml. M-$CaCl_2$ and 200 ml.

0·5 M-phosphate buffer (pH 6·8) to each litre of diluted starting material. These additions were made in small portions (using volumes in proportion to total) with vigorous mechanical stirring. After 30–45 min. the precipitate was filtered off by suction on a Buchner funnel, washed with water (without loss of enzyme), and eluted by grinding thoroughly in a mortar with 10 % (w/v) $(NH_4)_2SO_4$, 250 ml. being used for the precipitate from every litre of slowly with stirring. After standing overnight at 4° the flocculent precipitate was collected by filtration, with the aid of a little 'Hyflo Supercel' (Johns-Manville Ltd.). The solid on the filter was extracted several times with small volumes of borate buffer or distilled water. The extracts were usually dialyzed against several changes of borate buffer (pH 7·4) in which the enzyme is fairly stable. A typical preparation is shown in Table 1.

Table 1. *Stages and yields in the purification of collagenase*

Stage	Description	Volume (l.)	Q* units/ml.	κ* units/ml.	Le* units/ml.	Stage yield of collagenase (%): Calc. from Q-content	Stage yield of collagenase (%): Calc. from κ-content	Overall yield of collagenase (%): Calc. from Q-content	Overall yield of collagenase (%): Calc. from κ-content	Non-dialyzable nitrogen (NDN) (mg./ml.)	κ units/mg. NDN	κ/Le
A	Crude filtrate	180	2·1	3·2	3·4	—	—	—	—	0·283	11·3	0·94
	Pptd. with $(NH_4)_2SO_4$ (60 kg./100 l.). Scum dissolved in H_2O	21	11·6	29	22·8	64	106	64	106	1·19	24·4	1·27
B and C	First calcium phosphate treatment, giving Eluate I	7·87	23·1	44·6	5·8	75	57	48	61	0·12	374	7·7
	Treatment with charcoal (1·5% w/v)	7·87	19·5	39·2	3·8	85	88	41	54	0·051	770	10·3
	Second calcium phosphate treatment, giving Eluate II	3·9	23·3	62·5	—	59	79	24	42	0·046	1360	—
	Treatment with charcoal (0·8 %)	3·78	17·7	38·5	—	74	60	18	25	0·035	1100	—
	Dialyzed overnight	5·08	12·8	29	$<\frac{1}{2}$	97	101	17	26	—	—	—
D	Fractional precipitation with $(NH_4)_2SO_4$. Solution of ppt. dialyzed against borate buffer (pH 7·4)	0·084	604	1230	5·5	78	70	13	18	0·590	2100	224

* Q = collagenase enzyme units; κ = collagenase antigen units; Le = lecithinase antigen units.

diluted starting material. The precipitate was left with the eluent for several hours or overnight at 4°, before filtering. The precipitate was usually eluted again with half the previous volume of 10 % $(NH_4)_2SO_4$ and the two eluates combined (Eluate I).

Eluate I was stirred for 3–5 min. with an amount of Farnell's L.S. charcoal found to be suitable in a trial experiment (usually about 1 %), and filtered. The filtrate was then dialyzed overnight against running tap water. Sufficient solid $(NH_4)_2SO_4$ was then added to the dialysate to bring the concentration to 3·75 % (w/v) and the adsorption and elution with calcium phosphate repeated as before, to give Eluate II.

Eluate II was then dialyzed against running tap water (if it had retained too much colour it was first subjected to a second charcoal treatment as before). The dialysate was cooled in an ice-water bath and 2 vol. ice-cold 51 % (w/v) $(NH_4)_2SO_4$ solution added

Determination of optimum conditions for the various stages of the process

(i) *Adsorption on calcium phosphate (stage B)*

Adsorption of collagenase on calcium phosphate provided the best means of separating it from lecithinase, but the conditions which give the maximum separation consistent with good yields of collagenase are dependent on several factors as outlined below.

Formation of the calcium phosphate precipitate. The precipitate was formed *in situ*, as it was found that not only did aged calcium phosphate give much poorer yields of collagenase, but the separation from lecithinase was also much less efficient.

pH *of the buffer used in the precipitation.* 50 ml. starting material (28 Q units/ml.; 40 Le units/ml.; 3·2 % $(NH_4)_2SO_4$) at pH 6·3, were treated with 4 ml. M-$CaCl_2$ and 10 ml. 0·5 M-phosphate buffer at a range of pH values (see Fig. 1). At

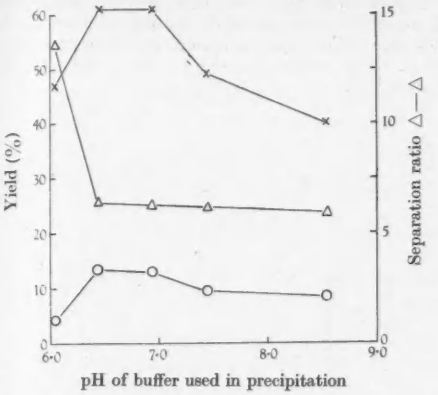


Fig. 1. The variation of yield of enzyme, and of efficiency of separation of collagenase from lecithinase, with the pH of the phosphate buffer used in the precipitation of calcium phosphate. The efficiency of separation of collagenase from lecithinase is measured by the separation ratio, i.e. the ratio Q units/Le units in the product to Q units/Le units in the starting material. Yield of collagenase enzyme in eluates ×—×. Yield of lecithinase antigen in eluates ○—○.

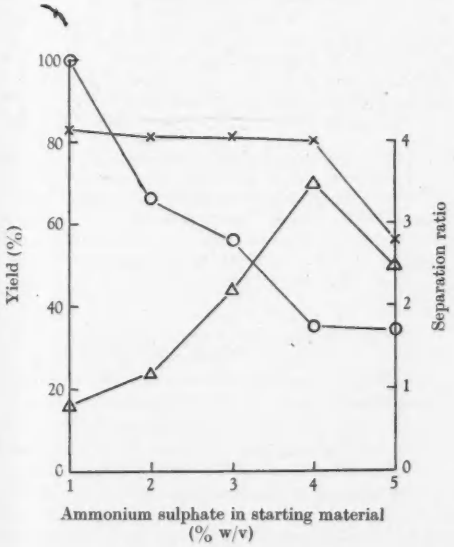


Fig. 2. The variation in yield of enzyme, and efficiency of separation of collagenase from lecithinase, with the concentration of ammonium sulphate in the starting material in the first calcium phosphate treatment. Separation ratio values and graphical symbols have the same significance as in Fig. 1.

pH 5·4 and below, no precipitate was formed. The precipitates were eluted with 50 ml. portions of 10% $(NH_4)_2SO_4$, and the eluates tested for collagenase and lecithinase content. Fig. 1 shows that the optimum pH appears to be 6·5–7·0, and in all further work 0·5 M-phosphate buffer at pH 6·8 was used. Variation of the pH of the starting material from pH 6·3 to 6·8 did not affect the result.

The concentration of $(NH_4)_2SO_4$ *in the starting material.* To 20 ml. portions of the enzyme preparations (*a*) or (*b*) (see below) were added varying amounts of $(NH_4)_2SO_4$ and water to 25 ml., 2 ml. M-$CaCl_2$ and 5 ml. 0·5 M phosphate (pH 6·8). Precipitates were eluted with 20 ml. 10% $(NH_4)_2SO_4$ and the eluates tested. Two preparations are described. Enzyme preparation (*a*): diluted starting material (11·5 Q units/ml.; 23·3 Le units/ml.; Q/Le = 0·49); see Fig. 2. Enzyme preparation (*b*): dialyzed eluate from a calcium phosphate treatment (Eluate I; 79 Q units/ml., 27 Le units/ml., Q/Le = 2·9); see Fig. 3.

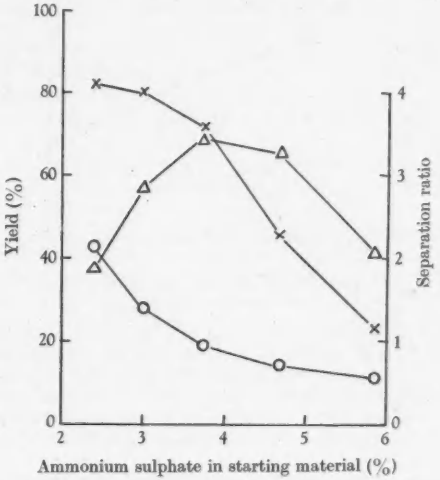


Fig. 3. The variation of yield of enzyme, and efficiency of separation of collagenase from lecithinase, with the concentration of ammonium sulphate in the starting material in the second calcium phosphate treatment. Separation ratio values and graphical symbols have the same significance as in Fig. 1.

The curves show that although the details differ for the two preparations, the optimum conditions are similar, and in all further work the concentration of $(NH_4)_2SO_4$ was adjusted to 3·75% (w/v) before the calcium phosphate treatment.

The concentration of enzyme in the starting material. Varying amounts of the enzyme preparations (*c*), (*d*) and (*e*) (see below) were diluted to the same volume, the concentration of $(NH_4)_2SO_4$ adjusted to 3·75% (w/v), and a calcium phosphate adsorption and elution carried out as usual. Enzyme preparation (*c*) was starting material derived from van Heyningen's medium (van Heyningen, 1948) (72 Q units/ml., 47 Le units/ml., Q/Le = 1·5) (see Fig. 4). Enzyme preparation (*d*) was starting material derived from meat-

broth medium (50 Q units/ml., 178 κ units/ml., 120 Le units/ml., Q/Le = 0·42) (see Fig. 5). Enzyme preparation (*e*) was a dialyzed eluate from a first calcium phosphate treatment (Eluate I) (75 Q units/ml., 250 κ units/ml., 22 Le units/ml., Q/Le = 3·4) (see Fig. 6).

The curves in Figs. 4 and 5 show that, for the first treatment with calcium phosphate, the recovery of collagenase and separation from lecithinase were both improved as the initial concentration of enzyme was decreased, and that

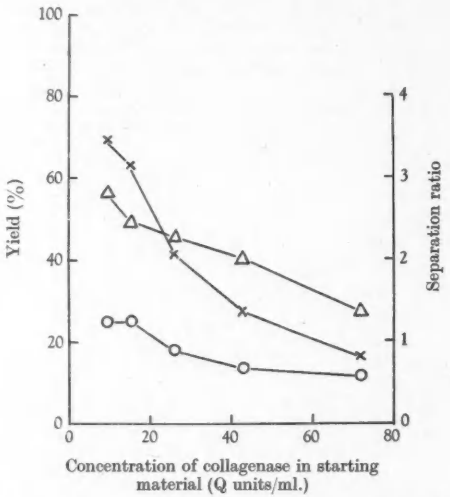


Fig. 4. The variation of yield of enzyme, and separation of collagenase from lecithinase, with the concentration of collagenase in starting material derived from van Heyningen's medium in the first calcium phosphate treatment. Separation ratio values and graphical symbols have the same significance as in Fig. 1.

despite differences in the starting materials of preparations (*c*) and (*d*) (see Q/Le ratio), the results were qualitatively similar. In order to minimize the inconvenience of working with large volumes, we compromised by diluting the starting material to a collagenase concentration of 12–15 Q units/ml. in all our subsequent routine preparations.

Fig. 6 shows that for the second treatment with calcium phosphate best yields and separation are again associated with low initial enzyme concentrations, but this is much less marked than for the first calcium phosphate treatment. In practice second calcium phosphate treatments were always carried out without dilution.

(ii) *Charcoal treatment (stage C)*

Much pigment is removed in the calcium phosphate treatments, but sufficient remains to be troublesome in the final concentrates. Farnell's L.S. charcoal will remove most of the pigment and allow twofold purification of collagenase with 80–100% yield. The charcoal treatment also has the advantage that lecithinase is adsorbed to a relatively greater extent than collagenase.

The eluate (containing 10% $(NH_4)_2SO_4$) from either a first or second calcium phosphate treatment is stirred for 3–5 min. with an appropriate amount of charcoal as determined by previous experiment (usually about 1%), and then filtered.

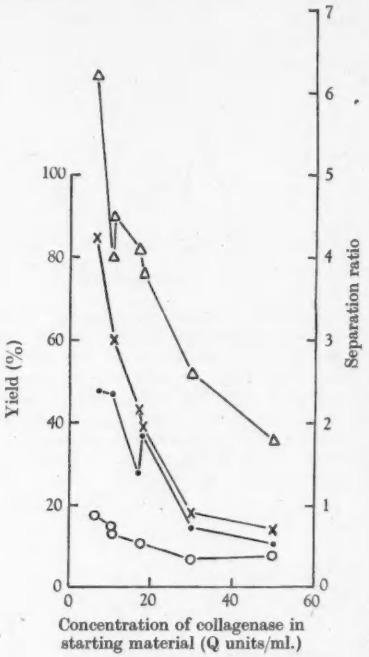


Fig. 5. The variation of yield of enzyme, and separation of collagenase from lecithinase, in starting material derived from meat broth medium in the first calcium phosphate treatment. Separation ratio values have the same significance as in Fig. 1. Yield of collagenase enzyme ×—×. Yield of collagenase antigen •—•. Yield of lecithinase antigen o—o.

Adsorption on hide powder

The enzyme can be adsorbed on hide powder and eluted from it, as shown by the following example:

75 g. of 60-mesh hide powder were added to 8·5 l. of dialyzed starting material (containing 49 Q units/ml.; 48 Le units/ml.), stirred for 1 hr., filtered, washed with 1 l. of water and eluted twice by stirring for 1 hr. with 500 ml. of buffer (M/30-phosphate, pH 7·4, containing 0·5% NaCl (w/v)). The eluates were collected by filtration, the hide powder returned to the original supernatant and the process repeated twice. The combined eluates (2·9 l., containing 43 Q units/ml., and 0·5–1 Le unit/ml.) contained the collagenase in 34% yield, and less than 1% of the original lecithinase.

The use of hide powder for routine purification was, however, abandoned because of difficulties of filtration on a large scale, the partial solution of hide powder and its cost.

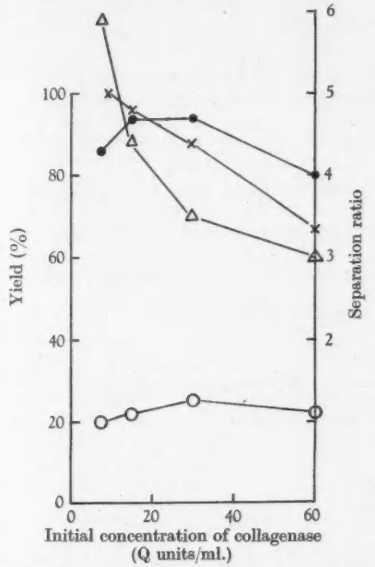


Fig. 6. The variation of yield of enzyme, and separation of collagenase from lecithinase, with the initial concentration of collagenase in a second treatment with calcium phosphate. Separation ratio values and graphical symbols have the same significance as in Fig. 5.

Properties of the purified enzyme

The purest preparation so far obtained (excluding material which has been subject to dialysis at pH 10·2—see below) contained 2600 Q units of collagenase/ml., 5800 κ-combining units of collagenase/ml., 33 Lε units of lecithinase/ml., ≯ 10 units of θ-toxin/ml., 2·27 mg. N/ml., i.e. κ units/mg. N = 2550. (Preparations containing 12,000 κ units/mg. N have been obtained after dialysis at pH 10·2.) The hyaluronidase activity of the preparation has not been tested. The enzyme acts optimally over a range of pH 6·0–7·5, is stable in borate buffers at alkaline and neutral pH, but is relatively unstable at acid pH. (A preparation kept at pH 4·0 for 4 hr. at 4° and then restored to pH 6·8 had lost nearly 40 % of its activity.) Pure preparations are not stable in phosphate buffers; e.g. equal volumes of a given preparation were dialyzed for 3 days at 4° against several changes of (*a*) Palitzsch borate buffer pH 8·0 (Clark, 1928) and (*b*) M/15-phosphate buffer pH 8·0 with respectively 90 and 47 % recovery of collagenase activity.

Dialysis of purified collagenase preparations against several changes of borate-Na_2CO_3 buffer pH 10·2–10·4 (Clark, 1928) resulted in a considerable reduction in the test dose against κ-antitoxin in azocoll tests, i.e. an increase in the κ-combining power units/ml., sometimes, but not always, accompanied by a corresponding increase in enzymic activity, i.e. rise in Q units/ml.

For example a preparation B 269 H (2320 Q units of collagenase/ml., 7140 κ-combining power units of collagenase/ml.) in equilibrium with borate buffer pH 7·4, was redialyzed to equilibrium against borate-Na_2CO_3 buffer pH 10·4; the dialysate was unchanged in volume and contained 4100 Q units of collagenase/ml., and 14,300 κ-combining power units of collagenase/ml., corresponding to an increase of about 75 % in the enzymic activity and 100 % in the combining power.

It is possible that the dialysis at pH 10·2–10·4 causes the formation of active enzyme from some inactive precursor which also has different immunological specificity.

Parallel electrophoretic investigations by Mr P. A. Charlwood are discussed in the addendum to this paper.

Purified enzyme preparations containing *c.* 20 Q units of collagenase/ml. are inactivated by heating at 80° for 5 min.

We have confirmed the observation of Maschmann (1938*a*) that collagenase is partially inactivated in 0·001 M-solutions of copper and mercury ions (the latter being most effective). Manganese, cobalt, and iron salts (after long contact with the enzyme) are all inhibitory to varying extents. The decrease in enzymic activity is not accompanied by any change in the combining power. Magnesium and zinc ions appear to be without effect on the enzymic activity.

Kinetic studies

pH *optimum.* Hide powder (850 mg.) was incubated 1 hr. at 37° with 10 ml. of a mixture of 10 ml. buffer and 2 ml. enzyme, using (*a*) veronal-acetate-HCl buffer (Michaelis, 1931); (*b*) M/15-phosphate buffer; (*c*) Palitzsch borate buffer (Clark, 1928). Determinations of total N were made on the filtrates. Very little variation in the extent of digestion of the hide powder was observed over the range pH 6·0–7·5 (in the case of borate buffer, the lowest pH used was 6·9). Similar results were obtained with gelatin.

Substrate concentration. Column D of Table 2 shows that at concentrations of hide powder higher than 750 mg./10 ml. there is very little further rise in filtrate nitrogen, and further work on the kinetics was done with 850 mg./10 ml. owing to the difficulty in working with higher concentrations. It can be seen from column E (Table 2) that the amount of hide powder dissolved in the controls (heat inactivated enzyme) is approximately a constant percentage of the total amount present over the range 250–1000 mg./10 ml.

Table 2. *The optimum concentration of substrate for the action of collagenase on hide powder*

(Final collagenase concentration 0·28 Q units/ml. Buffer: Palitzsch borate pH 7·4.)

Hide powder concentration (mg./10 ml.)	Nitrogen content of filtrate (mg. N/10 ml.)		Nitrogen content of filtrate due to enzyme action (mg. N/10 ml.) (B − C)	Hide powder dissolved in controls* (%) $\left(\frac{C}{A} \times \frac{10^4}{15{\cdot}4}\right)$
	When active enzyme used	When heat-inactivated enzyme used		
A	B	C	D	E
100	8·4	3·7	4·7	24·0
250	17·0	7·6	9·4	19·8
500	28·8	14·0	14·8	18·2
750	40·3	20·6	19·7	17·9
1000	49·4	29·4	20·0	19·1

* In a separate experiment it was found that the hide powder used contained 15·4% nitrogen.

Table 3. *The optimum concentration of substrate for the digestion of gelatin by hide powder*

N (mg./ml.) in gelatin substrate solution	Mixture incubated			Nitrogen content of filtrate (mg. N/100 ml.)			Col. G recalculated to refer to 80 ml. gelatin (mg. N)
	Gelatin (ml.)	Buffer* (ml.)	Enzyme† (ml.)	When active enzyme used	When heat-inactivated enzyme used	Due to enzyme action (E − F)	
A	B	C	D	E	F	G	H
5·57	10	1·0	0·25	3·27	0·37	2·90	23·2
2·73	20	2·0	0·50	5·55	0·29	5·26	21·0
1·37	40	4·0	1·00	7·09	0·42	6·67	13·3
0·69	80	8·0	2·00	8·50	0·53	7·97	8·0

* Palitzsch borate pH 7·2.
† Active enzyme contained 21 Q units of collagenase/ml.

In determining the substrate concentration optimum for gelatin, the mixtures, made up as shown in Table 3, were incubated 1 hr. at 37°. Then sufficient ice-cold buffer (Palitzsch borate pH 7·2 in which all the solutions had been prepared) was added to bring the final volume to 90 ml., followed by 10 ml. precipitant (5% (w/v) phosphotungstic acid in N-H_2SO_4). Owing to the difficulty of preparing standard solutions of gelatin, the concentrations of the gelatin solutions used are indicated in column A of Table 3 by their nitrogen content. The values of mg. N/100 ml. filtrate due to the enzyme activity are shown in column G, Table 3. Column H gives the calculated values for the total amount of nitrogen 'soluble' under the given conditions (i.e. when 1 vol. of precipitant is added to 9 vol. of a mixture containing *c.* 0·6 mg. N/ml.) which would be produced by 2 ml. of the enzyme solution used, acting on 80 ml. of gelatin of the concentration given in column A in a total volume of 90 ml. A final gelatin concentration during incubation corresponding to about 5 mg. gelatin N/ml. seems to be about optimal.

Activity curves. Hide powder: see Fig. 7, curve *A*. M/15-phosphate buffer (pH 6·5), 850 mg. hide powder/10 ml., and incubation for 1 hr. at 37° were used.

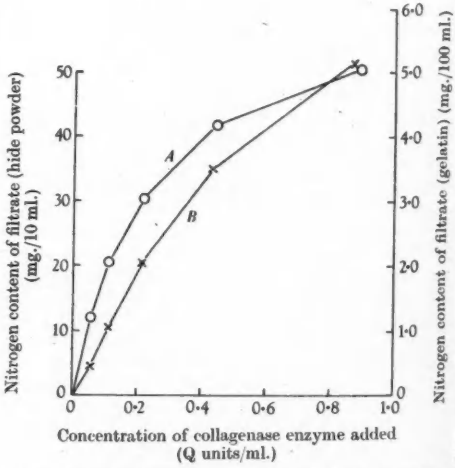


Fig. 7. Collagenase activity curves. *A*, with hide powder; *B*, with gelatin as substrates.

Gelatin: see Fig. 7, curve *B*. 10 ml. amounts of a solution of gelatin (5 mg. N/ml.) in borate buffer (pH 7·2) were incubated with amounts of enzyme

as shown (total vol. during incubation, 11·25 ml.). After 1 hr. at 37°, 78·75 ml. ice-cold buffer was added, followed by 10 ml. precipitant; remainder of the procedure as usual. Controls: heat-inactivated enzyme treated in exactly the same way.

Interpretation of activity curves. Bergmann (1933) found that the enzymic digestion of gelatin sheets, the area of which remained constant during digestion, could be described by the formula: $x = kt\sqrt{E}$, where x = percentage digestion; k = constant; t = time; E = percentage enzyme concentration, and that the formula also held for the early part of the digestion, of fibrous collagen, i.e. these reactions followed the Schutz rule. He suggested that this could be explained by the assumption that the digestion of collagen fibres proceeds only from their ends. Fig. 8

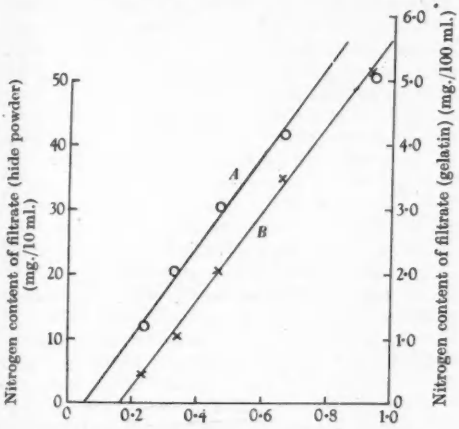


Fig. 8. Data from activity curves replotted to show variation in activity with the square root of the enzyme concentration. *A*, hide powder; *B*, gelatin.

shows that the digestion of hide powder and gelatin (in solution) by low concentrations of *Cl. welchii* collagenase in a given time also proceeds approximately according to a similar expression, $x = k\sqrt{E} - a$, where a is a constant, possibly due to partial inactivation of the enzyme during the course of the incubation, and that the kinetics of digestion of undissolved hide powder and dissolved gelatin are similar. Therefore we feel that Bergmann's suggestion that collagen fibres are digested only from the ends needs re-examination.

Substrate specificity

Proteins other than collagen or gelatin. 5 ml. of substrate solution or suspension (containing about 2% protein) were incubated with 1 ml. enzyme solution for 1 hr. at 37°, then 10 ml. 0·3M-trichloroacetic acid were added, and the solution filtered through Whatman no. 3 filter papers. Controls: 5 ml. substrate, 10 ml. 0·3M-trichloroacetic acid added, then 1 ml. enzyme, filtered. The extent of digestion was estimated by either 'tyrosine' or total N determination on filtrates.

(*a*) Urea-denatured haemoglobin, prepared according to Anson (1938): little or no effect in the absence of cysteine, slight digestion in the presence of cysteine.

(*b*) Casein (British Drug Houses Ltd. L.W.S.): little or no effect.

(*c*) Normal horse serum: no effect.

(*d*) Urea-denatured normal horse serum: little or no effect.

(*e*) Heat-denatured horse serum albumin: no effect in the absence of cysteine; in presence of cysteine, about 10% of the substrate was digested. The experiment was repeated with a more highly purified preparation of collagenase, which showed no activity against heat-denatured horse serum albumin, even in the presence of cysteine and during 20 hr. incubation at 37°.

(*f*) Clupein sulphate: no action even during 20 hr. in the presence of cysteine.

The final collagenase concentration in experiments (*a*)–(*d*) was 16 Q units/ml., and in (*e*) was 7 Q units/ml. For comparison, it may be calculated from the data in Table 2, that collagenase in a final concentration of 0·28 Q units/ml. digests nearly 25% of the hide powder present (in 2·5% suspension) in 1 hr. at 37°.

Titration of groups liberated by the action of collagenase on gelatin. After incubation of gelatin with collagenase samples were titrated as follows:

(1) For amino nitrogen by the method of Pope & Stevens (1939).

(2) By the KOH-ethanol method (Grassmann & Heyde, 1929).

(3) By the HCl-acetone method (Linderstrøm-Lang, 1928).

In the control experiment a corresponding amount of heat-inactivated enzyme was used, the values were subtracted from those obtained where the active enzyme was used, and the result expressed in terms of m.equiv. of $-NH_2$ or $-COOH$ formed by the enzyme in the whole mixture incubated (50 ml.). The conditions of the experiment were such that method (2) should measure the total acids formed, and method (3) the total $-NH_2$ groups formed. (For a critical study of these methods, see Richardson, 1934.) Method (1) provides a check on the others. The experiment was carried out on duplicate mixtures. The results (Table 4) were consistent with the splitting of a peptide bond.

Possible synthetic substrates. The following substances were found to be unaffected by collagenase: hippuramide, L-glycylproline, L-carbobenzyloxyprolylglycine, pyroglutamic acid, *O*-acetyl-L-hydroxyproline, *ON*-diacetyl-L-hydroxyproline.

Table 4. *The* $-NH_2$ *and* —COOH *groups liberated by the action of collagenase on gelatin*

(50 ml. gelatin solution (final concentration of gelatin N, 5·6 g./l.) with sodium chloride, 1% (w/v) incubated at 37° for 20 hr. with collagenase (1 Q unit/ml.) or the corresponding amount of heat-inactivated enzyme. All solutions adjusted to pH 7·0 before mixing. Toluene (2 drops) added to each mixture.)

Method of estimation	m.-equiv. of $-NH_2$ or —COOH formed
Pope & Stevens's (1939) copper method	1·90 ($-NH_2$)
HCl-acetone titration	2·08 ($-NH_2$)
KOH-ethanol titration	2·14 (—COOH)

Neutralization by antibody

It was shown by Oakley *et al.* (1946) that antisera produced in horses in response to immunization with culture filtrates of *Cl. welchii* type A generally contained antibody which inhibited the action of collagenase on collagen in the form of azocoll and collagen 'paper'. It was therefore to be expected that such antisera would neutralize the gelatin-splitting activity of our enzyme preparations. However, not only could inhibition not be demonstrated satisfactorily, but in some cases mixtures containing excess antiserum were apparently more active than when no antiserum was present.

Example: Varying amounts of antiserum R 8537 (from the same batch as the standard serum R 8531 of Oakley *et al.* (1946) and defined as containing 180 anti-κ units/ml.) were added to 1·0 ml. enzyme preparation (26 Q units/ml., 102 κ-combining units/ml.) diluted in sufficient borate buffer (pH 7·4) to make the final volume 7·5 ml. After standing 0·5 hr., 5 ml. amounts of the mixtures were incubated with 40 ml. gelatin solution (0·73 mg. N/ml.) for 1 hr. at 37°. 5 ml. of the usual precipitant were added and total N determinations were carried out on the filtrates. The controls consisted of a parallel series of mixtures, using heat-inactivated enzyme, which were incubated in exactly the same way.

The results are given in Table 5. All the mixtures were tested for activity against azocoll by the incubation overnight at 37° of 0·5 ml. of mixture with 1 ml. 'azocoll-manucol' suspension (Oakley *et al.* 1946). The results are shown in Table 5, column F. As expected, only the first mixture gave a strongly positive reaction (+ +), while all the others were completely negative. Mixtures 2 and 3 were undoubtedly over neutralized as far as activity against azocoll is concerned. The failure to neutralize the activity against gelatin might be due to one of several causes:

(*a*) Part, if not all, of the 'gelatinase' activity might be due to an immunologically distinct enzyme. If this were so, the 'gelatinase' would appear to be a very poor antigen, since several antisera having very varied contents of other antibodies have been shown to be ineffective in neutralizing this activity against gelatin.

(*b*) Dissociation of the complex formed by the enzyme with the homologous antibody. This appears an unlikely explanation in view of the fact that the final concentration of antiserum was over 50 anti-κ units/ml. and the incubation time only 1 hr., while the usual concentration in testing for combining power (κ-unitage) is less than 1 anti-κ unit/ml. and the time of incubation overnight.

(*c*) The anti-collagenase antibody may prevent gross particles of hide powder, but not the comparatively small molecules of gelatin, from reaching the catalytic centre of the enzyme. It has previously been observed that in some cases enzyme-anti-enzyme complexes are formed which retain all the activity of the uncombined enzyme, for example, the work of Campbell & Fourt (1939) on catalase.

None of the above explanations accounts for the rise in activity which has sometimes been observed in the presence of serum. That this is not due to any direct action of the serum on gelatin is shown by the controls (5 and 6, Table 5); the rise shown (over control 4) is probably due only to non-precipitable nitrogenous material in the added serum, and is in any case so small as to be negligible in the present discussion.

Table 5. *The effect of serum containing anti-collagenase antibody on the action of a collagenase preparation on gelatin*

Mixture no.	Added to 40 ml. gelatin: κ units of collagenase	Added to 40 ml. gelatin: Anti-κ units of serum	Nitrogen content of filtrate (mg. N/50 ml.)	Nitrogen content of filtrate produced by active enzyme (mg. N/50 ml.)	Activity of mixtures against azocoll
A	B	C	D	E	F
1	68	0	4·64	4·23	+ +
2	68	240	6·96	6·27	No detectable activity
3	68	360	8·29	7·39	"
4	<1/3	0	0·41	—	"
5	<1/3	240	0·69	—	"
6	<1/3	360	0·90	—	"

DISCUSSION

According to the theories of Bergmann and his colleagues (Bergmann & Fruton, 1941) the specificity of proteolytic enzymes is governed by the nature and arrangement of the amino-acid residues on either side of the peptide bond that is hydrolyzed. The arrangement of amino-acids to which a given enzyme is specific may not occur in certain proteins, in which case those proteins would not be attacked by the enzyme; but it is surprising that out of the number of proteins tested the appropriate conditions of specificity for the collagenase of *Cl. welchii* appears to be present only in collagen (and its derivative, gelatin). This protein differs from other proteins in its high content of proline and hydroxyproline, but the narrow specificity of collagenase does not appear to be connected with these amino-acids. The failure of the enzyme to hydrolyze glycylproline or carbobenzyloxyprolylglycine indicates that the enzyme is neither a prolidase (Bergmann & Fruton, 1937) nor a prolinase (Grassmann, Schoenebeck & Auerbach, 1932).

We were prepared to consider the possibility that collagenase is not a peptidase, but that it dissolves collagen and liquefies gelatin as a result of its catalyzing some reaction other than the hydrolysis of a peptide bond. For example, it is conceivable, as Dr A. Neuberger suggested to us, that collagen and gelatin might contain ester linkages between the —OH groups of hydroxyproline residues and those carboxyl groups of glutamic acid that are not involved in peptide bonds. Hydrolysis of such linkages by an esterase might result in the solution of collagen or the liquefaction of gelatin. For this reason we tested the action of our enzyme preparations on *O*-acetyl-L-hydroxyproline and on *ON*-diacetyl-L-hydroxyproline, but no hydrolysis resulted.

On the other hand, the enzyme does appear to catalyze the hydrolysis of peptide bonds since amino-acids are liberated by it from gelatin, as is evident from our results. (Maschmann (1937) estimated his gelatinase by titrating the liberated amino groups.) The production of free amino groups is very slow compared with the solution of collagen or the liquefaction of gelatin.

The increase in κ-combining units of collagenase antigen (i.e. the decrease in the κ test dose), which occurred when partially purified preparations of the enzyme were dialyzed against borate-Na_2CO_3 buffer (pH 10·2–10·4), was confirmed on several occasions. It was often accompanied by an increase in enzyme activity (as measured by a rise in Q units/ml.). The enhancement of the combining power and enzymic activity was retained on redialyzing to equilibrium against pH 7·4 borate buffer. It is possible that the dialysis at pH 10·2–10·4 causes the formation of collagenase from some inactive precursor, which also has different immunological specificity. The parallel electrophoretic investigations described by Mr P. A. Charlwood in the addendum to this paper lend some support to this view, since a comparison of the electrophoretic properties (at pH 7·4) of a preparation before and after an intermediate dialysis at pH 10·2 (in borate-Na_2CO_3) shows that this treatment has produced some irreversible change in the protein constitution of the mixture.

SUMMARY

1. The preparation, from culture filtrates of *Cl. welchii* type A, of a proteolytic enzyme, 'collagenase' (κ-toxin), which appears to attack only collagen and gelatin, is described. It has been obtained substantially free from the α- and θ-toxins of *Cl. welchii* and purified (with respect to non-dialyzable nitrogen) about 200-fold.

2. The partially purified enzyme is optimally active over the pH range 6·0–7·5, and is stable in solution in borate but not in phosphate buffers.

3. An increase in activity and antibody-combining power after dialysis against borate buffer at pH 10·2 was observed.

4. Sera neutralizing the activity of collagenase-containing filtrates against collagen do not inhibit their activity against gelatin.

5. The electrophoretic properties of partially purified preparations are discussed in an addendum.

We are grateful to Dr R. G. Macfarlane and Dr J. D. MacLennan for suggesting this problem. We also wish to thank Miss G. H. Warrack for many immunological tests, Miss A. G. Kennedy for many nitrogen and other determinations, Mr G. A. Spiby for much technical assistance, Mr J. C. Macsween and the staff of the toxin laboratories for the provision of the crude filtrates, Dr S. Wilkinson of the Wellcome Chemical Research Laboratories for synthesizing the various peptides, etc., mentioned and Dr A. C. McDougall of the Wellcome Chemical Works for the preparation of 120-mesh hide powder. We are grateful to Dr C. L. Oakley for many helpful discussions.

Addendum. Electrophoresis of Collagenase Preparations

By P. A. CHARLWOOD

Experimental. The electrophoresis of collagenase preparations was carried out in a Tiselius apparatus at +0·5°, the diagonal schlieren method being employed. Preparations were dialyzed against borate or borate-carbonate buffer at the required pH. The buffer was renewed every 12 hr., and equilibrium was considered to be attained when no difference could be detected between the refractive indices of fresh buffer and buffer which had been in contact with the sample for 12 hr. Potential gradients employed in the U-tube ranged from 2·7 to 3·1 V./cm.

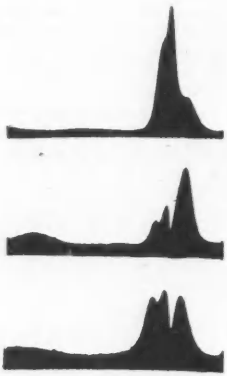
The collagenase preparation B 285B was dialyzed at pH 7·4 and a run carried out (see Fig. 9A). The material recovered from the U-tube (unavoidably diluted with buffer) was combined with the rest of B 285B, and filtered. The filtrate (B 286A) was dialyzed against buffer at pH 10·2; the dialysate (B 286B) was unchanged in volume and Table 6 shows that this treatment resulted in a large increase (over 200%) in the total κ units present. Preparation B 286B was submitted to electrophoresis (see Fig. 9B). Again the residues were collected (B 287A), dialyzed back to pH 7·4 (B 287B) without significant change in volume and Table 6 shows that there was no loss in κ-unitage during the return to pH 7·4. A final run was then carried out on preparation B 287B (see Fig. 9C).

Results of electrophoretic experiments. The electrophoretic pattern of B 285B (Fig. 9A) shows three peaks. The pattern of B 286B (Fig. 9B) also shows three peaks, but is considerably different, as the fastest-moving component now constitutes the major portion of the protein. The final material, B 287B (Fig. 9C) is clearly different from B 285B. These two, being at the same pH, should be almost identical if the sequence of pH changes had not affected the material present. The fastest-moving component in B 287B is present in much greater proportion than in B 285B.

The above series of changes in the curves correlates with the alteration in combining power. It would appear that the combining power is associated

Table 6. *Collagenase preparations used in the electrophoretic experiments*

Sample	Q units/ml.	κ units/ml.	mg. N/ml.	κ units/mg. N	θ units/ml.	Le units/ml.
B 285B	3,200	7,000	3·22	2,180	<1	37·8
(B 286A)	(2,220)	(5,100)	—	—	—	—
B 286B	3,000	11,100	1·79	6,200	—	—
(B 287A)	(2,360)	(7,140)	—	—	—	—
B 287B	2,050	7,500	1·37	5,500	—	—



	pH	Buffer	Potential gradient (V./cm.)	Time between starting current and exposure (min.)
A	7·4	Borate	3·10	335
B	10·2	Borate-carbonate	2·68	192
C	7·4	Borate	3·02	230

Fig. 9. Electrophoresis of collagenase. Anode (ascending) limb curves are shown. Migration is from left to right.

chiefly or wholly with the fast component. It would be of great interest to separate electrophoretically a pure specimen of this component and to ascertain whether or not this is the case. The chief difficulty lies in achieving a sufficiently good separation of pure material from the amounts of the preparations available.

Mobility determinations were made at pH 10·2 and 7·4. The results are shown in Table 7. They are uncorrected for buffer viscosity, and are also liable to contain errors caused by the inability to maintain a very steady current, owing to large fluctuations of the mains supply.

Table 7. *Mobilities of components present in collagenase preparations, expressed in cm./sec./V./cm.* $\times 10^{-5}$

pH	Component		
	Fast	Middle	Slow
10·2	−7·29	−5·96	−5·41
7·4	−5·25	−4·37	−3·76

The above results have been confirmed qualitatively by runs on other batches of collagenase preparations.

REFERENCES

Anson, M. L. (1938). *J. gen. Physiol.* **22**, 79.
Bergmann, M. (1933). *Nature, Lond.*, **131**, 698.
Bergmann, M. & Fruton, J. S. (1937). *J. biol. Chem.* **117**, 189.
Bergmann, M. & Fruton, J. S. (1941). *Advances in Enzymology*, **1**, 63–96. New York: Interscience Publishers, Inc.
Campbell, D. H. & Fourt, L. (1939). *J. biol. Chem.* **129**, 385.
Clark, W. M. (1928). *The Determination of Hydrogen Ions*, 3rd ed. London: Baillière, Tindall and Cox.
Evans, D. G. (1945). *Lancet*, **249**, 478.
Grassmann, W. & Heyde, W. (1929). *Hoppe-Seyl. Z.* **183**, 32.
Grassmann, W., Schoenebeck, O. & Auerbach, G. (1932). *Hoppe-Seyl. Z.* **210**, 1.
Linderstrøm-Lang, K. (1928). *Hoppe-Seyl. Z.* **173**, 32.
Macfarlane, R. G. & MacLennan, J. D. (1945). *Lancet*, **249**, 328.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Maschmann, E. (1937). *Biochem. Z.* **295**, 1.
Maschmann, E. (1938*a*). *Biochem. Z.* **295**, 351.
Maschmann, E. (1938*b*). *Biochem. Z.* **297**, 284.
Maschmann, E. (1938*c*). *Biochem. Z.* **300**, 89.
Michaelis, L. (1931). *Biochem. Z.* **234**, 139.
Oakley, C. L., Warrack, G. H. & van Heyningen, W. E. (1946). *J. Path. Bact.* **58**, 229.
Pope, C. G. & Stevens, M. F. (1939). *Biochem. J.* **33**, 1070.
Richardson, G. M. (1934). *Proc. roy. Soc.* B, **115**, 121.
Robb-Smith, A. H. T. (1945). *Lancet*, **249**, 362.
Sen, S. N. (1942). *Ind. J. med. Res.* **30**, 335.
van Heyningen, W. E. (1948). *Biochem. J.* **42**, 127.

The Colorimetric Determination of Stilboestrol and Dienoestrol

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A preliminary account has been given (Warren & Goulden, 1945) of investigations that have been carried out on methods proposed for chemical assay of synthetic oestrogens. Two methods were studied. One (Dingemanse, 1939) is based upon the development of a red colour by the oestrogen in the presence of antimony pentachloride; the other (Malpress, 1945) relies on the measurement of the yellow colour obtained by nitration of the oestrogen followed by neutralization. The antimony reaction proved to be very sensitive. By its use the instability of dienoestrol in solution was demonstrated. On the other hand, stilboestrol (4:4′-dihydroxy-αβ-diethylstilbene) appears to be quite stable in solution in a variety of solvents. It was subsequently found (Wilder Smith & Williams, 1945) that the oestrogenic activity of solutions of dienostrol shows a similar decrease with time. Hence, chromogenic activity towards antimony pentachloride and oestrogenic activity appear to run parallel. On the other hand, solutions of dienoestrol that show very low activity in the antimony pentachloride reaction continue to react strongly in Malpress's test.

In the present paper details of these experiments are given, together with the results of some attempts to apply the antimony reaction to the estimation of synthetic oestrogens in urine of subjects treated therapeutically with these substances.

EXPERIMENTAL

The antimony pentachloride reaction

In the original description of this reaction (Dingemanse, 1939), few details are given. Stilboestrol dissolved in chloroform (washed free from ethanol) was treated with a 50 % (v/v) solution of antimony pentachloride in the same solvent. Difficulty was experienced in obtaining reproducible results by this simple technique, and a systematic study of the factors influencing colour development was undertaken.

(*a*) *Specificity.* Many stilbene derivatives develop colour in the presence of antimony pentachloride dissolved in ethanol-free chloroform (small amounts of ethanol change, or destroy, the colour). In Table 1 the results of qualitative tests on a range of substituted stilbenes are shown. The presence of at least one ethylenic bond appears to be essential for chromogenic power (hexoestrol gives practically no colour), but the colour is modified by the presence of substituent groups in the molecule. In general, the presence of a hydroxyl group tends to deepen the colour towards the red end of the spectrum. Weight for weight, stilboestrol develops more colour than does dienoestrol. For quantitative measurement of stilboestrol and of dienoestrol (using a Spekker photoelectric absorptiometer) Ilford Spectrum Green filter no. 604 was found to be the most suitable.

(*b*) *Solvent.* The preliminary experiments were carried out in chloroform (B.P. grade) which had been thoroughly washed with water to remove the small quantity of ethanol normally incorporated. The chloroform was dried over calcium chloride and distilled before use. Good colour development was obtained on mixing a chloroform solution of antimony pentachloride with a chloroform solution of the oestrogen, but the colour faded rapidly. In order to overcome this difficulty other chlorinated solvents were tried. Carbon tetrachloride was unsuitable since, although a red colour developed rapidly, there was almost complete precipitation of a red compound. Trichloroethylene also had to be excluded since it reacts with antimony pentachloride and develops colour. Ethylene dichloride was finally chosen as being the most suitable of the solvents tried. Good colour development occurs and the colour is much more stable than in chloroform. An additional practical advantage is that a good commercial grade of this solvent can be used without preliminary purification. Previous washing with water or with alkali followed by drying and distillation effected no improvement in colour stability.

Table 1. *Colour reactions of substituted stilbenes with antimony pentachloride*

Substance	Colour
Hexoestrol ($HO.C_6H_4.CHEt.CHEt.C_6H_4.OH$)	Transient yellow
Dienoestrol ($HO.C_6H_4.C(:CHMe).C(:CHMe).C_6H_4.OH$)	Red-brown
Stilboestrol ($HO.C_6H_4.CEt:CEt.C_6H_4.OH$)	Red
4:4′-Dihydroxy-$\alpha\beta$-dimethylstilbene	Red
4:4′-Dihydroxy-α-*n*-propylstilbene	Orange
4:4′-Dihydroxy-α-methyl-β-*n*-propylstilbene	Red
4:4′-Dihydroxy-α-ethyl-β-cetylstilbene	Red
4-Methoxy-$\alpha\beta$-diethylstilbene	Red-green
Stilbene	Yellow
α-Ethylstilbene	Brown
Triphenylethylene	Yellow-brown
α-(*p*-Hydroxyphenyl)stilbene	Brown-orange
Triphenylmethylethylene	Olive-green
4:4′-Diacetylstilbene	No colour
4:4′-Diacetyl-$\alpha\beta$-diethylstilbene	No colour

(*c*) *Concentration of antimony pentachloride.* The intensity of the final colour depends on the concentration of antimony pentachloride present. In order to establish the conditions for maximal colour development, 1 ml. of a solution of stilboestrol was treated with 9 ml. of solutions of antimony pentachloride of varying strengths. The results of these experiments are shown in Fig. 1. It will be seen that maximum colour is obtained when the final antimony pentachloride concentration lies between 1 and

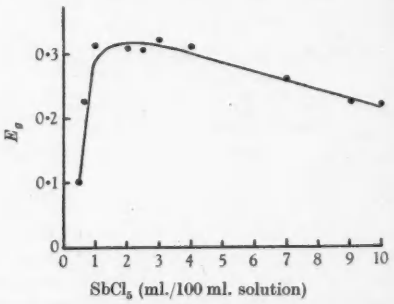

Fig. 1 Stilboestrol-$SbCl_5$ reaction. Influence of variation in concentration of $SbCl_5$ on the final intensity of colour. E_g is the extinction of the solution in green light.

4% (v/v). A reagent containing 3 ml. of antimony pentachloride dissolved in 100 ml. of ethylene dichloride was finally adopted as standard. For estimations of stilboestrol or dienoestrol, 9 ml. of this reagent were added to 1 ml. of a solution of the oestrogen in ethylene dichloride. Ordinary reagent quality antimony pentachloride (B.D.H.) was found quite satisfactory without any special purification, but the solution in ethylene dichloride employed as reagent should be freshly prepared immediately before use.

(*d*) *Time.* With dienoestrol, maximum colour development occurs immediately on mixing with the reagent. With stilboestrol the colour increases during

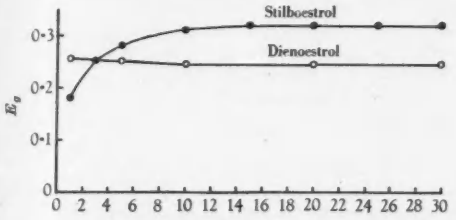

Fig. 2. Stilboestrol and dienoestrol colour reaction with $SbCl_5$. Effect of time of development on the final intensity of colour. E_g as in Fig. 1.

the first 10 min. and then reaches a maximum. The influence of time on colour development is illustrated in Fig. 2. As a routine, colour measurements were made 15 min. after mixing the oestrogen with the reagent.

(*e*) *Temperature.* The rate of development of maximal colour varies with temperature. Although the temperature coefficient is not large it was found convenient to standardize the conditions by maintaining the solutions at 30° during the 15 min. period of colour development.

The Malpress nitro reaction

A comparison was made of this method (Malpress, 1945) with the antimony pentachloride method. A detailed account of the application of the method has been given by Malpress, and only points of major interest will be mentioned here.

(*a*) *Specificity.* The nitro reaction (a general one for phenols) is comparatively non-specific, both as regards substances giving the reaction and as regards colour developed.

(*b*) *Spectral absorption characteristics and sensitivity.* In Fig. 3 are shown approximate light absorptions (measured on a Hilger Spekker absorptiometer fitted with Ilford Spectrum filters) of oestrogens treated with antimony pentachloride and of dienoestrol treated according to Malpress's procedure. It will be seen that, whereas the antimony reagent develops a red colour with a definite maximum absorption in the green, the nitro reaction leads to an orange colour showing rather general absorption at the violet end of the spectrum.

An Ilford Spectrum blue-green (no. 603) filter gave the highest values and most sensitive slope to the calibration curve for the nitro reaction. When this filter is used the sensitivity of the nitro-reaction determination is about one twentieth that of estimations by the antimony reagent.

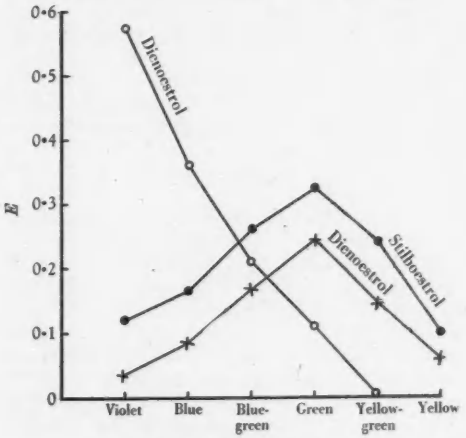

Fig. 3. Comparison of $SbCl_5$ reaction (stilboestrol and dienoestrol) and the nitro reaction (dienoestrol). Light absorption curves of the coloured products of these reactions. $SbCl_5$ reaction, oestrogen concentration 2 μg./ml. Nitro reaction, oestrogen concentration 20 μg./ml.

Application of the antimony pentachloride reaction to the determination of synthetic oestrogens in chloroform and in ethylene dichloride solutions

Since the ultimate objective of this work was the devising of a method for colorimetric determination of synthetic oestrogens in extracts prepared from human urine, the behaviour of these substances in solvents suitable for urine extraction (e.g. chloroform and ethylene dichloride) was studied. Since extraction of oestrogens from urine would involve their exposure to air, light, and normal room temperatures, no attempt was made to protect the oestrogen solutions from such influences.

Stock solutions of stilboestrol, dienoestrol, and *iso*dienoestrol were prepared and allowed to stand in colourless glass flasks under ordinary laboratory conditions. Exposure to direct sunlight was avoided. Samples were withdrawn, initially and at intervals, and their chromogenic power towards the antimony reagent determined. The initial value, determined as soon as possible after solution had been achieved, was taken as indicating the full chromogenic power

of the oestrogen initially present. Later values were recorded as percentages of this initial reading. The curves shown in Fig. 4 were constructed from mean value, whereas the antimony reagent showed only about 15% of dienoestrol to exist unchanged at this time.

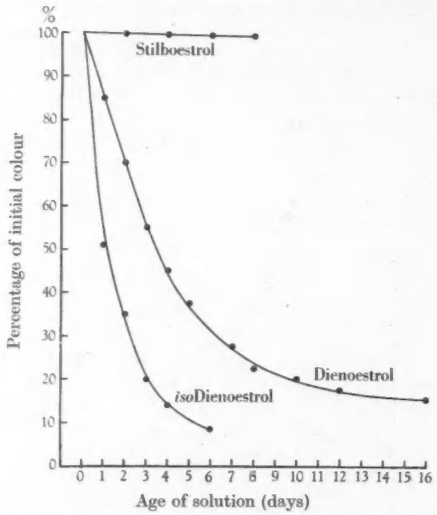

Fig. 4. Effect of age of solution of synthetic oestrogens on the reaction with $SbCl_5$. Chromogenic power expressed as percentage of initial colour value.

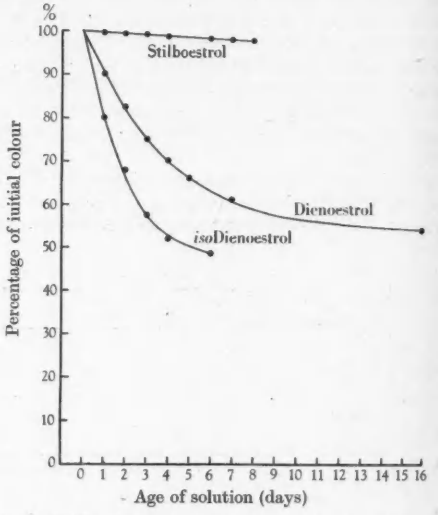

Fig. 5. Effect of age of solution of synthetic oestrogens on the Malpress nitro reaction. Chromogenic power expressed as percentage of initial colour value.

values determined in a number of separate experiments. It is clear that dienoestrol and *iso*dienoestrol in solution rapidly lose their chromogenic power, while that of stilboestrol is maintained at its initial level for a comparatively long period. The stability of dienoestrol was tested in a number of solvents, and in every case the chromogenic power towards the antimony reagent decreased with increasing age of the solution.

Application of Malpress's nitro reaction to the determination of synthetic oestrogens in chloroform and in ethylene dichloride solution

Simultaneously with the above experiments, the chromogenic power of the ageing stock solutions of stilboestrol, dienoestrol, and *iso*dienoestrol was measured by Malpress's method. Samples were withdrawn at intervals, the solvent evaporated at low temperature in a stream of nitrogen and the residue dissolved in glacial acetic acid. The subsequent procedure was as described by Malpress. The mean results of these experiments are shown in Fig. 5. The decrease in colour developed with increasing age of the solutions in the case of dienoestrol and of *iso*dienoestrol is again clearly shown, although the actual decrease at any given age is not so great as with the antimony reagent. The lowest value for dienoestrol (at 16 days) was 53·5% of the initial

Mechanism of the 'decay' of dienoestrol solutions

It appeared probable that the explanation of the gradual loss of reactivity of dienoestrol towards antimony pentachloride might be a slow oxidation of the substance leading to disappearance of ethylenic double bonds. The integrity of these bonds is apparently essential for chromogenic power.

In order to test this possibility, solutions of dienoestrol in ethylene dichloride were prepared, and stored in the light and at room temperature but in the absence of air, achieved by thorough evacuation of the tubes containing the solution and subsequent sealing when all air had been displaced by solvent vapour. The same number of control (unsealed) tubes was stored under the same conditions. One sealed tube and one control tube were tested with antimony reagent at weekly intervals.

At the end of the experiment (21 days) no loss of chromogenic power had occurred in the dienoestrol in the sealed tubes, whereas the unsealed controls had shown the usual rapid 'decay'. It can be concluded, therefore, that the decay is caused by atmospheric oxidation of the oestrogen.

Application of the antimony reaction to the estimation of synthetic oestrogens in extracts of human urine

It was clear, from the experiments described above, that the problem of the detection and estimation of dienoestrol in human urine was likely to

be a difficult one. The difficulties involved in the prevention of oxidation during the necessary hydrolytic and extraction processes appeared formidable. On the other hand, the proved stability of stilboestrol, and the high sensitivity of the reagent towards this oestrogen, suggested that it should be possible to obtain at least approximate measurements of the urinary excretion of stilboestrol in subjects undergoing treatment with this substance. Attention was therefore confined to urines likely to contain stilboestrol.

The complicating factor in the use of the antimony reagent on urine extracts is the presence of relatively large quantities of phenols. Small quantities of simple phenols (phenol or cresol) completely inhibit colour development. It was found, however, that the quantity of phenol present in the urine extracts could easily be reduced below the interference level by heating the extracts at 100° *in vacuo*. Recovery experiments with known amounts of stilboestrol (1–10 μg.), to which phenol or cresol had been added in amounts (5–10 mg.) comparable to those encountered in urine extracts, gave excellent results. The method finally adopted for investigating the stilboestrol content of urine was as follows:

(*a*) *Unhydrolyzed urine.* Fresh urine (100 ml.) was extracted with chloroform, the chloroform washed with water, dried, and distilled off *in vacuo*. The residue was treated as described below for the extract from hydrolyzed urine.

(*b*) *Hydrolyzed urine.* Urine (100 ml.) was acidified with 15 ml. of conc. HCl, 50 ml. of benzene added and the mixture refluxed for 3 hr. on the water bath. The benzene layer was then removed and replaced by a fresh 50 ml., and the mixture again refluxed for 3 hr. The combined benzene extracts were washed twice with water, the benzene separated, filtered and evaporated.

The residue was dissolved in 10 ml. of ethylene dichloride. 1 ml. of this solution was removed and diluted to 10 ml. with ethylene dichloride. This 10 ml. of solution served as a 'blank' for the colorimetric estimation; the urine extracts frequently had a red colour which contributed to the final red colour developed by the oestrogen, and it was necessary to correct for this.

A further 1 ml. of the original solution was transferred to a pyrex Thunberg tube which was then immersed in a boiling water bath and the solvent carefully evaporated at ordinary pressure. A vacuum was then applied (water pump) and the tube maintained in the bath for 15 min. The top part of the tube was carefully heated with a free flame to prevent condensation of the phenolic distillate.

After cooling the tube, the contents were dissolved in 1 ml. of ethylene dichloride and treated with 9 ml. of antimony reagent. The colour was allowed to develop at 30° for 15 min. and then measured (against the blank solution described above).

Urines from ten different patients were examined by this method. Eight of these subjects were receiving oral therapeutic doses of 5–15 mg. of stilboestrol daily. Two subjects had been implanted subcutaneously with 100 mg. of stilboestrol. In none of the extracts (unhydrolyzed or hydrolyzed) was unequivocal evidence of the presence of stilboestrol obtained. The colour developed was in all cases anomalous (i.e. brownish instead of red). On the other hand, small quantities of stilboestrol added to urine (either in solution in organic solvents or in aqueous solution as the sodium derivative) before hydrolysis and extraction could be recovered with approximately 100 % accuracy. It is possible that the brown colour developed in these extracts may be due to decomposition products of stilboestrol present in the urines, since it was not observed in control experiments with normal urines.

DISCUSSION

Stilboestrol and dienoestrol are both widely used as therapeutic oestrogenic agents. In clinical practice dienoestrol is often used in preference to stilboestrol. This preference appears to be based on the belief that dienoestrol causes fewer side effects (nausea and vomiting) than does stilboestrol. Although this may be so when the two oestrogens are administered orally in the same amounts it is by no means certain that under these conditions the same oestrogenic effect is obtained.

The most important result of the present work is the demonstration of the great difference in stability between stilboestrol and dienoestrol. While the former substance has considerable stability in solution and resists oxidation, solutions of dienoestrol undergo very rapid atmospheric oxidation. This oxidation leads to loss of chromogenic potency towards both the Dingemanse and the Malpress reagents, and at the same time there is a corresponding decrease in oestrogenic potency. Since this oxidative destruction occurs readily in a variety of solvents (including aqueous solvents) it would clearly be difficult to prepare solutions of dienoestrol of known oestrogenic potency for therapeutic purposes unless some antioxidant were incorporated in such solutions.

The attempts to demonstrate the presence of stilboestrol in the urine of subjects undergoing treatment with relatively large amounts of this oestrogen, which have been described above, must be regarded as preliminary experiments. However, if account is taken of the high sensitivity of the method used, the total amount of stilboestrol (conjugated or free) in these urines must have been very small. The urine extracts gave anomalous colours (brown). But if the total absorption of green light by such solutions is taken as representing the maximum amount of stilboestrol which could be present (an obvious overestimate), the total daily urinary excretion of stilboestrol in patients receiving a daily oral dose of

15 mg. did not exceed 5 % of the dose given. Since this figure is, admittedly, an overestimate, the results indicate that, broadly speaking, none of the administered stilboestrol was present in the extracts. This does not, of course, imply that the urine did not contain oestrogenic substances. It is possible that metabolites of stilboestrol were present, still possessing oestrogenic activity, but whose structure precluded the formation of red-coloured products with antimony pentachloride. The formation of the anomalous brown pigments by such urine extracts may be significant in this connexion.

The negligible amounts of free stilboestrol found in the extracts of unhydrolyzed urine in the present experiments are possibly to be expected by analogy with other compounds and with the results of experiments with stilboestrol in other species. The main excretory product might well be a conjugated product. Some observations of Malpress are of interest in this connexion. Malpress (private communication) has so far found it impossible to estimate the ether-insoluble forms of stilboestrol in cow's urine, owing to the complete destruction of this oestrogen when hydrolysis by hydrochloric acid takes place *in the presence of other urinary constituents*. On the other hand, as he has shown (Malpress, 1946), hydrolysis of the glucuronide by hydrochloric acid *in simple solution* gives recoveries of the oestrogen which closely approximate those which can be calculated from postulates as to the action of hydrochloric acid on pure stilboestrol. The failure to demonstrate conjugated stilboestrol in the present experiments may be due to similar causes.

SUMMARY

1. The behaviour of stilboestrol, dienoestrol, and *iso*dienoestrol in Dingemanse's antimony pentachloride reaction and Malpress's nitro reaction has been examined.

2. In solution in a variety of solvents, stilboestrol is stable, but dienoestrol and *iso*dienoestrol are unstable. This instability of dienoestrol and of *iso*-dienoestrol in solution arises from their susceptibility to rapid atmospheric oxidation.

3. Attempts to demonstrate the presence of free or conjugated stilboestrol in the urine of subjects undergoing treatment with large therapeutic doses of this oestrogen failed to reveal the presence of significant amounts.

One of us (F. L. W.) is indebted to the Sir Halley Stewart Trust for a Fellowship and to the Anna Fuller Fund for a grant. Generous grants to the Chester Beatty Research Institute from the British Empire Cancer Campaign greatly assisted the work. We should also like to thank our colleagues on the Chemical Advisory Panel of the Committee on Oestrogens in Cancer (under the aegis of the Royal Society of Medicine and the British Empire Cancer Campaign) for much helpful advice and discussion. We are particularly grateful to Prof. E. C. Dodds, Dr W. Lawson, and Dr W. F. Short for the gift of compounds and to Dr Freda Herbert for supplying some of the urine extracts.

REFERENCES

Dingemanse, E. (1939). *Acta brev. neerl. Physiol.* **9**, 118.
Malpress, F. H. (1945). *Biochem. J.* **39**, 95.
Malpress, F. H. (1946). *Nature, Lond.*, **158**, 790.
Smith, A. E. Wilder & Williams, P. C. (1945). *Nature, Lond.*, **156**, 718.
Warren, F. L. & Goulden, F. (1945). *Biochem. J.* **39**, xliv.

The Osmotic Pressure of Insulin Solutions

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Sjögren & Svedberg (1931) examined solutions of crystalline insulin in the ultracentrifuge. From determinations of the sedimentation constant they concluded that it is a homogeneous protein, stable between pH 5 and 7. From sedimentation equilibrium measurements they calculated a molecular weight of 35,000. Polson (1937) determined the diffusion constant of insulin, and calculated from this ($D_{20} = 8{\cdot}2 \times 10^{-7}$) and Sjögren & Svedberg's sedimentation constant ($S_{20} = 3{\cdot}47 \times 10^{-13}$) the molecular weight 40,900. From Crowfoot's (1938) crystallographic measurements the weight of the dry unit cell is about 36,000.* The unit cell has trigonal symmetry, and the molecular

* The value quoted by Crowfoot (1938) for the dry unit cell weight is 37,600. This has been calculated on the assumption that the moisture contents of air-dried insulin crystals are 5·35 %. Miller & du Vigneaud (1937) pointed out the difficulties of obtaining accurate values for the moisture contents of insulin crystals and found 10·2 % as the result of repeated analyses. The present author analyzed five samples of air-dried insulin crystals and found variations between 10 and 12 % for the moisture contents. Completely dried crystals, if left at room temperature, absorbed water until they contained from 10 to 11 %.

weight of insulin in the crystal is therefore 36,000 or 12,000.

The molecular weight of insulin was generally accepted to be about 36,000 up to the publication of the work of Miller & Andersson (1942), who carried out a thorough study of the sedimentation and diffusion constant and calculated the molecular weight 46,000 from their data ($S_{20} = 3{\cdot}55 \times 10^{-13}$ and $D_{20} = 7{\cdot}53 \times 10^{-7}$).

Gutfreund & Ogston (1946) re-examined the sedimentation constant with a view to observing any changes of insulin in alkaline solutions and on dilution. They found that $S_{20} = 3{\cdot}35 \times 10^{-13}$ over a range of pH from 6·8 to 9·6 and at protein concentrations from 0·1 to 1·0 %. From this sedimentation and the diffusion constant ($D_{20} = 7{\cdot}0 \times 10^{-7}$) obtained by Gutfreund (unpublished) the molecular weight 46,600 can be calculated.

On account of the discrepancies between the data for the molecular weight ranging from 35,000 to 46,000, a detailed study of the osmotic pressure of insulin solutions was undertaken. No previous osmotic pressure measurements have been recorded for this protein, probably because of the sparing solubility near the isoelectric point and the indication of dissociation outside the pH range 4·5–7·5 (Sjögren & Svedberg, 1931).

Osmotic pressure and membrane potentials

The results of osmotic pressure measurements were used for the calculation of the molecular weight of insulin. As shown by Adair & Robinson (1930), this can be done by the use of the following equation:

$$P - P_i = P_p = RTg_p M_p,$$

where P = observed osmotic pressure; P_i = ion pressure difference; P_p = pressure due to protein, i.e. colloid osmotic pressure; g_p = osmotic coefficient (correcting for repulsive and attractive forces); and M_p = mol. protein/l. of solvent. The correction factors can usually be eliminated simultaneously by use of the equation

$$M = 10RT/\pi_0,$$

where M = molecular weight; $\pi_0 = P/C$ extrapolated to zero concentration; C = concentration of protein in g./100 ml. of solution. (The factor 10 in the above equation brings this up to conc./l. of solution.)

If the charge of the protein particles in solution is high and the salt concentration of the solvent buffer low, P_i may still be significant at low-protein concentrations. This has to be checked by membrane potential measurements or chemical analysis of diffusible ion concentration on both sides of the membrane (Adair & Robinson, 1930; Adair & Adair, 1934). For the work described in this paper the membrane potential was used for the calculation of P_i after the equation developed by Adair (1928):

$$P_i = 15{\cdot}4\, E^2\, \Sigma C_i'' Z_i^2,$$

where P_i is the ion pressure difference in mm. of mercury, E is the membrane potential, and $\Sigma C_i'' Z_i^2$ is the sum of the buffer ion concentration in the dialysate, multiplied by the square of their valence. All the experiments described in this paper were carried out on solutions of protein concentration below 1 %, and under those conditions the factor g_p can be neglected for nearly spherical molecules, such as insulin. Throughout this paper we have therefore used the simple formula $P_p = P - P_i = RT\,(C/M)$ for molecular weight calculations.

EXPERIMENTAL

Two types of osmometer were used for the experiments described in this paper. The simple apparatus developed by Adair (1925) was employed for the first two series of measurements. The osmotic pressure is read directly from the height of the solution in the capillary. When equilibrium is reached the capillary tube is removed and two samples of protein are taken from the membrane. The first is used for capillarity measurements and the second for estimation of protein concentration. Wherever required, membrane potential measurements were then carried out on the remaining solution. The technique described by Adair & Adair (1934) was followed in every detail for the measurement of membrane potentials.

The corrections for capillarity in the experiments recorded in Tables 1 and 2 are between 13 and 20 mm., depending on the inner diameter of the capillary. A study of the duplicate measurements of capillarity suggested that it would not be possible to get accurate results from osmotic pressure experiments on solutions containing less than 0·5 % protein, as a small error in the correction would have a large effect on the molecular weight calculation. If very wide capillary tubes are used the correction can be reduced, but equilibration is prolonged considerably. A new osmometer, employing toluene as pressure head in the capillary, has been developed by Mr G. S. Adair (to be published). With this apparatus much more reliable capillarity corrections can be made. The osmometers have been used according to the technique developed by Adair (personal communication), and the results of measurements on dilute solutions are recorded in Tables 3 and 4.

The following insulin preparations were used: (1) crystalline insulin from Boots Pure Drug Co. Ltd., (2) Squibb crystalline insulin. Nitrogen, moisture and ash contents of all samples of insulin received were examined. For the nitrogen estimation the micro-Kjeldahl procedure, with all precautions suggested by Chibnall, Rees & Williams (1943), was used. This analysis was important as the concentration of insulin in solutions after osmotic pressure measurements were calculated either from nitrogen contents or from refractive index difference (Δn) measurements. Δn was estimated in a Zeiss interferometer which was standardized with insulin solutions, their concentration previously being determined from micro-Kjeldahl nitrogen. The values obtained for the nitrogen contents of three samples of Boots crystalline insulin and one sample of Squibb crystalline insulin were all within 1 % of the value quoted by Chibnall *et al.* (1943), i.e. 15·4 % nitrogen in moisture- and ash-free protein.

The samples of protein were the same as those used by Gutfreund & Ogston (1946) for their ultracentrifuge experi-

Table 1. *Molecular weight of Boots crystalline insulin in buffer* pH 7·0

(In 0·1 M-phosphate; temp. 18°.)

P_p corrected to 0° (mm. H_2O)	Protein conc. (%)	Mol. wt.
9	0·14	36,000
9	0·145	35,300
11	0·18	37,800
14	0·21	34,700
14	0·22	36,400
	Mean	36,060 ± 1080 (S.D.)

ments and shown to be homogeneous. Examination in the Tiselius electrophoresis apparatus also confirmed their purity.

RESULTS

The major difficulty encountered during all the experiments on insulin solutions was the low solubility and with that the measurement of very small osmotic pressures. The first series of measurements was carried out on solutions of insulin in 0·1 M-phosphate buffer (pH 7·0) at 18°. Though it was possible to obtain supersaturated solutions, containing 1% protein, under these conditions precipitation occurred during osmotic equilibrium, and the final protein concentration was always below 0·25%. Table 1 shows the results of this first series of measurements, giving a mean molecular weight for insulin of 36,000. The membrane potential under those conditions of protein and buffer concentration was measured and found to be negligible

$$(P_i < 0{\cdot}05 \text{ mm. } H_2O).$$

It was observed that, on reducing the phosphate concentration of the buffer from 0·1 to 0·01 M, solutions containing up to 0·8% protein could be obtained at pH 7·0 and a temperature of 0°. Table 2 shows the results of a series of experiments carried out under those conditions. As can be seen the membrane potential effect, and with that the excess pressure of diffusible ions, becomes significant, both on account of higher protein concentration and of the reduced salt concentration in the buffer. The standard deviation from the mean molecular weight, 47,700 ± 3000, is much larger than that obtained from measurements on ovalbumin and lactoglobulin (Gutfreund, 1944, 1945), but this is due to the relatively small pressures measured and also due to the correction for P_i which, changing with the square of the membrane potential, may account for much of the deviations.

It was thought possible that the difference between the molecular weight calculated from measure-

Table 2. *Molecular weight of crystalline insulin preparations in buffer* pH 7·1

(In 0·01 M-phosphate; temp. 0°.)

Preparation	P (mm. H_2O)	P_i (mm. H_2O)	P_p (mm. H_2O)	Protein conc. (%)	Mol. wt.
Boots	23	2	21	0·46	51,000
	42	9	33	0·64	45,000
	35	7	28	0·58	48,000
	43	10	33	0·70	49,000
	36	7	29	0·58	46,000
	47	12	35	0·77	51,000
	40	8	32	0·60	43,000
	34	7	27	0·58	50,000
Squibb	51	13	38	0·77	47,000
	35	3*	32	0·65	47,000
				Mean	47,700 ± 3000 (S.D.)

* This experiment was carried out in 0·04 M-phosphate buffer pH 7·1.

Table 3. *Molecular weight of Squibb crystalline insulin (temperature* 0·05°)

P_p (mm. H_2O)	Protein conc. (%)	Mol. wt.
A. In phosphate buffer (0·02 M, pH 7·0).		
6·4	0·112	40,800
9·2	0·154	38,800
8·8	0·164	42,800
7·7	0·126	38,200
6·5	0·116	41,200
8·4	0·146	40,300
7·7	0·132	38,800
	Mean	40,300 ± 1400 (S.D.)
B. In phosphate buffer (0·02 M, pH 7·5).		
7·6	0·137	41,700
7·1	0·115	37,800
10·0	0·180	41,500
	Mean	40,300 ± 1800 (S.D.)

Table 4. *Molecular weight of Squibb crystalline insulin*

(In 0·02 M-phosphate buffer pH 7·0; temp. 20·5°.)

P_p (mm. H_2O, corrected to 0°)	Protein conc. (%)	Mol. wt.
8·1	0·122	34,900
9·6	0·136	30,500
9·4	0·143	35,400
9·8	0·115	27,100
10·2	0·132	39,800
10·2	0·143	32,200
10·8	0·159	34,100
	Mean	32,000 ± 2800 (S.D.)

ments on solutions between 0·5 and 0·8 % protein and on solutions containing 0·15–0·25 %, may be due to dissociation of insulin on dilution. The accuracy of the results of Table 1 can be disputed on account of the difficulties of capillarity measurements in the type of osmometer used. The toluene osmometer, mentioned above, was therefore employed for a series of experiments which is recorded in Table 3. These results indicate, first, a dissociation of insulin on dilution, and secondly, that no further dissociation takes place on changing the pH from 7·0 to 7·5. As a further test for dissociation of insulin in dilute solutions, a series of measurements was carried out under identical conditions of pH and buffer and protein concentration, at a higher temperature. Table 4 shows the record of a number of experiments carried out at 20·5°. A marked increase of dissociation on raising the temperature can be observed.

DISCUSSION

In solutions containing 0·5–1·0 % protein the molecular weight of insulin is ~48,000. This is shown both by the results of osmotic pressure measurements and combined diffusion and sedimentation constants. In dilute solutions, containing less than 0·25 % protein, the molecular weight is lower, indicating dissociation. A further proof of the dissociation phenomena is given by the decrease of mean molecular weight with temperature. It has not been possible to upset the equilibrium completely to the side of the dissociation product and so to determine the size of the smaller unit, but both crystallographic evidence (Crowfoot, 1938) and chemical evidence (Chibnall, 1946) would be consistent with the assumption that subunits of molecular weight 12,000 are loosely bound to form insulin molecules. Further investigations on the molecular weight of the dissociation product will be described in a subsequent paper.

On the assumption that the dissociation of insulin on dilution is a reversible reaction $A_4 \rightleftharpoons 4A_1$ (A_4 is a molecule of weight 48,000 and A_1 a molecule of weight 12,000), the results recorded in Tables 3 and 4 are used for the calculation of the concentration of the A_4 and A_1 units, from the mean molecular weight at 0·5 and 20·5° respectively. Though particles of the type A_2 (molecular weight 24,000) and A_3 (molecular weight 36,000) may also be present, a correct measure of the number of bonds broken between these subunits is obtained by considering the equilibrium $A_4 \rightleftharpoons 4A_1$ only.

If x is the concentration of A_4 units and $(1-x)$ the concentration of A_1 units, the equilibrium constant $K = \dfrac{x}{(1-x)^4}$, x is calculated from the equation

$$\frac{1}{M} = \frac{x}{M_1} + \frac{(1-x)}{M_2},$$

where M = mean molecular weight, M_1 = molecular weight of A_4 unit, M_2 = molecular weight of A_1 unit. The free-energy change is calculated from

$$\Delta G = -RT \ln K:$$

at 0·5°: $x = 0{\cdot}93$, $K = 38{,}730$, $G = -57{,}292$ cal.
at 20·5°: $x = 0{\cdot}83$, $K = 994$, $G = -40{,}166$ cal.

Van't Hoff's reaction isochore can be used for the calculation of the total heat change ΔH, from the two equilibrium constants:

$$\ln \frac{K_1}{K_2} = -\frac{\Delta H}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right),$$

$$\Delta H = -29{,}106 \text{ cal.}$$

From the relation $\Delta H = \Delta G + T\Delta S$ we can work out the entropy change ΔS. Taking a round mean value for $\Delta G = -48{,}000$ cal. (at 283° absolute),

$$\Delta S = +67 \text{ cal./degree.}$$

This is a first attempt to study reversible dissociation of proteins in relation to the energies involved. Though it is not justifiable to lay too much stress on the values obtained for ΔG and ΔH, they do give some indication of the type of reaction involved. This method of approach to the study of dissociation reactions is being continued and expanded for insulin and other proteins showing similar effects.

It is very likely that the dissociation effect, demonstrated here, accounts for the variation in the figures for the molecular weight of insulin, previously reported. Any measurements carried out in solutions would vary with concentration. The most interesting effect is the apparent formation of molecules consisting of three units (of molecular weight 12,000) in the crystal, while in solution the molecule can consist of up to four units. It is, of course, possible that still larger aggregates may form at higher concentrations. The calculation of molecular weight from crystallographic data is, under favourable conditions, the most accurate available, and it is a genuine difference in size of the molecule and not a disagreement of results, which is suggested.

Gutfreund & Ogston (1946) could not find any signs of dissociation in their ultracentrifuge experiments at concentrations below 0·2 %, but it is obvious that such small amounts as 10 or even 20 % of dissociation product would not be visible in the sedimenting boundary of such dilute solutions.

SUMMARY

1. It is shown that the molecular weight of insulin, calculated from osmotic pressure measurements on solutions containing 0·5–0·8 % protein at pH 7·0, is 48,000 ± 3000.

2. Insulin tends to dissociate on dilution to below 0·25 % protein concentration. The dissociation effect increases with temperature.

3. Some preliminary calculations, on the energies involved in the dissociation reaction, have been carried out.

4. A possible cause for the discrepancies between previously reported molecular weight values is discussed.

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REFERENCES

Adair, G. S. (1925). *Proc. Roy. Soc.* A, **108**, 627.
Adair, G. S. (1928). *Proc. Roy. Soc.* A, **120**, 573.
Adair, G. S. & Adair, M. E. (1934). *Biochem. J.* **28**, 199.
Adair, G. S. & Robinson, M. E. (1930). *Biochem. J.* **24**, 1864.
Chibnall, A. C. (1946). *J. int. Soc. Leath. Chem.* **30**, 1.
Chibnall, A. C., Rees, M. W. & Williams, E. F. (1943). *Biochem. J.* **37**, 354.
Crowfoot, D. (1938). *Proc. Roy. Soc.* A, **164**, 580.
Gutfreund, H. (1944). *Nature, Lond.*, **153**, 406.
Gutfreund, H. (1945). *Nature, Lond.*, **155**, 237.
Gutfreund, H. & Ogston, A. G. (1946). *Biochem. J.* **40**, 432.
Miller, G. L. & Andersson, K. J. L. (1942) *J. biol. Chem.* **144**, 459.
Miller, G. L. & du Vigneaud, V. (1937). *J. biol. Chem.* **118**, 101.
Polson, A. (1937). Thesis: University of Stellenbosch.
Sjögren, B. & Svedberg, T. (1931). *J. Amer. chem. Soc.* **53**, 2657.